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Investigations Concerning Hydrolysis and Stabilization of Antiradiation Compounds

ANNUAL REPORT

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Antiradiation compounds: ethiofos; WR 2721; WR 1065, hydrolytic and thermal properties; encapsulation; drug dosage forms; blood plasma assay; pharmacokinetics.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report contains results of the following studies:

1. Encapsulation of ethiofos (WR 2721) for use as oral dosage forms.
2. In vitro evaluation of the most promising samples.
3. Development of analytical methodology for the active species.
4. Preliminary pharmacokinetic results of in vivo studies.

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SUMMARY

This report covers studies conducted on S-2-(3-aminopropylamino)ethyl phosphorothioic acid, ethiofos (WR 2721), over the period January 16, 1983 through January 13, 1984 which includes portions of the third and fourth years of the contract. The most significant results obtained during this report period are the following.

1. Plasma HPLC assay methods have been developed for ethiofos over the range of 0.05 µg/mL to >1000 µg/mL. The initial method developed was presented at a technical meeting and has been published. An improved method has also been presented at a technical meeting and have been published.

2. The improved ethiofos assay method has been used successfully in two dosing experiments with beagle dogs. Preliminary evaluation of the results indicates the data is compatible with a recycling process with a clearance of about 0.3 L/hr/kg and an apparent terminal half-life of about 1.5 days.

3. An HPLC plasma assay method has been developed for WR 1065 over the range of 1 µg/mL to >500 µg/mL. The method has been presented at a technical meeting and has been submitted for publication.

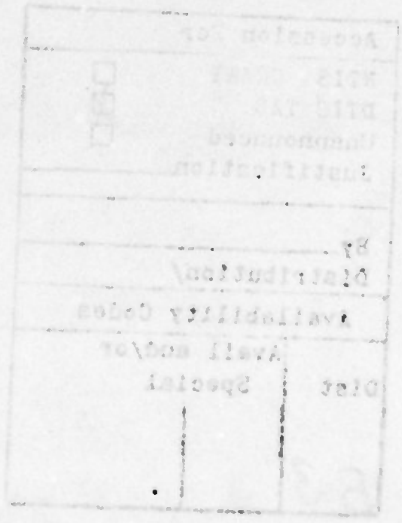
4. WR 1065 disappears quickly in dog plasma even at -78°C. Methods to stabilize the plasma samples have been investigated and some appear very promising.

5. The WR 1065 assay method has been used in a preliminary ethiofos dosing study with a beagle dog. Results indicate relatively low levels of WR 1065 in the plasma and none was detected after 720 minutes post infusion. In light of WR 1065 instability in plasma these results should be treated as tentative.

FOREWORD

Citations of organizations and tradenames do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].



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I. STATEMENT OF PROBLEM

Over a period of several years, the U.S. Army Medical Research and Development Command has been actively pursuing the development of a drug or combination of drugs which could be taken by military personnel for protection from the effects of the ionizing radiations from a nuclear weapons attack. Several chemical compounds (when dosed intravenously in animal studies) were found to be promising, in particular the phosphorothioates. When administered IV the best of these materials, S-2-(3-aminopropylamino)ethyl phosphorothioic acid, ethiofos, has been shown to protect mice, dogs, and Rhesus monkeys against X-ray and/or gamma radiation and to protect mice against neutron radiation. However, oral dosing of ethiofos failed to protect either dogs or monkeys and produced vomiting in dogs.

In an attempt to explain the lack of activity following oral administration, it has been postulated that ethiofos is readily hydrolyzed to the thiol in the stomach of the animal species and that the thiol is poorly absorbed. (Credence has been given to this hypothesis as a result of studies completed during the first year of this program which showed that ethiofos was readily hydrolyzed at a pH of 1.0). If such is the case, then it would be appropriate to protect ethiofos with an enteric coating for passage through the stomach. One convenient method of applying such a coating is microencapsulation, a process which may subject the drug to elevated temperatures. Therefore, prior to undertaking any microencapsulation studies, adequate thermal stability of ethiofos had to be established.

During the first year term of the contract, ethiofos was shown to be thermally stable and was successfully encapsulated as microspheres and as microcapsules. Several different matrices were established which protected the drug from acid hydrolysis yet would release it in solutions of pH 7.5. These formulations appear promising as the initial candidates for oral dosing studies. However, prior to these studies a plasma assay for ethiofos had to be established and during the period covered by the Annual Report dated January 1983, an assay for ethiofos was developed such that animal dosing studies could proceed.

After the assays were completed for the initial dosing studies, it became apparent that greater sensitivity in the detection of ethiofos was required and therefore an improved assay method was developed.

In addition an assay method for WR 1065, the thiol metabolite of ethiofos, was needed and studies initiated during this report period resulted in the establishment of an assay.

As a result of the development of the assays, oral and further IV dosing studies with ethiofos can now proceed.

II. BACKGROUND

During the period covered by the first, second and third annual reports under this contract, investigations were conducted which demonstrated the following:

- the hydrolytic instability of ethiofos under acidic conditions but greater stability under alkaline conditions;
- the thermal stability of ethiofos when heated at 60°C under nitrogen for at least one hour;
- the successful encapsulation of ethiofos in a variety of glycerides, fatty acids and paraffins and mixtures thereof;
- the stability of ethiofos in certain encapsulated products in pH 1.0 solutions at 37°C for 1.5 hours;
- the release of ethiofos from certain of the promising encapsulated forms at pH 7.5 (37°C for 2 hours) using a buffered solution or synthetic intestinal fluid;
- the ability to directly assay microcapsules and some buffered solutions for ethiofos using an HPLC procedure developed at SwRI;
- the ability to analyze for ethiofos present in synthetic intestinal fluid using an alternate procedure which removes interferences in this system;
- the development of a plasma assay method for ethiofos over the range of $\leq$1 µg/mL to >1000 µg/mL;
- the successful application of the assay method in three ethiofos dosing experiments with dogs;
- the reproducibility of the encapsulation of ethiofos to give good protection of the drug against acid hydrolysis and rapid release at pH 7.5.

These results continue to justify the approach taken; that is, the development of an acceptable oral dosage form by encapsulation of the drug with enteric-type coatings which protect it from acid hydrolysis during passage through the stomach and then release the drug in the intestinal tract.

In order to develop and evaluate such a dosage form, the reported studies were undertaken.

III. EXPERIMENTAL

A. Analytical Methods Development

1. Apparatus

a. HPLC Unit A

A Waters Associates Model 244 HPLC fitted with a Rheodyne 7125 injector, RCM-100 Radial Compression Module with a 5μ C-18 analytical column and LDC Fluoromonitor III fluorescence detector comprised the flow system of Unit A. Excitation and emission filters were 370 nm and 418-700 nm, respectively. Peak integration was accomplished using a Waters Associates "Data Module".

b. HPLC Unit B

An IBM Model LC/9533 HPLC equipped with a Rheodyne 7125 injector was fitted with a Whatman Partisil 5-ODS-3 analytical column, Bioanalytical Systems LC-4B amperometric detector and mercury/gold transducer cell, and an Omniscribe strip chart recorder to form HPLC system unit B.

c. HPLC Unit C

(1) Mode C-1 (For 1065 Assay)

An LDC Constametric III HPLC pump equipped with a Rheodyne 7125 injector was fitted with a BAS Biosphere ODS 5μ analytical column, Bioanalytical Systems LC-4B amperometric detector and mercury/gold transducer cell, and an Omniscribe strip chart recorder to form HPLC system unit C-1.

(2) Mode C-2 (For Ethiofos Assay)

For ethiofos assay use, Unit C was modified by using a RCM-100 Radial Compression Module containing a 5μ C-18 analytical column, LDC Fluoromonitor III fluorescence detector, and a LDC/Milton Roy CI-10 Integrator and printer.

2. Solvents

Organic solvents were Baker Analyzed HPLC grade. Water was purified with a Millipore Milli RO4 purification system.

3. Reagents

All reagents were ACS reagent grade. Tetrabutylammonium hydroxide was purchased from Aldrich Chemical Company.

B. In Vivo Studies

The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards as set forth by the "Guide for the Care and Use of Laboratory Animals" DHEW Publication, NIH 74-23.

Healthy, male beagle dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, Michigan and used in pilot dosing studies to test the analytical method.

Dog dosing studies No. 4, 5, 6, 7, and 8 were conducted on 1/19/83, 2/14/83, 10/4/83, 10/26/83, and 12/14/83, respectively, and the protocols and logs are attached in Appendix F. Assays of samples from studies No. 5, 6, 7 and 8 are presented in the Results Section. Assay of samples from study No. 5 was not completed because of a lack of sufficient sensitivity of the then-current method and because it was discovered that storage of samples at -20°C did not offer adequate protection.

C. Microencapsulation Studies

1. Materials

The active materials being investigated in the encapsulation studies in progress are:

- Ethiofos Lot No. BK 45589 AP-10-25
- WR 2823 Lot No. AEAN 25574

The glycerides, fatty acids and paraffin used for the shell coatings were standard food grade and were listed in the Annual Report dated January 1983, for the project.

2. Procedures

The centrifugal extrusion process as described in the Annual Report dated January 1982 for the project was used to prepare the microspheres and microcapsules.

3. Evaluation

The hydrolytic stability and release rate studies of the capsules were conducted in accordance with the methods described in the Annual Report dated January 1982.

IV. RESULTS

A. Analytical Methods Development

1. Ethiofos (WR 2721)

The following publications and presentations at technical meetings occurred during this report period as a result of the ethiofos assay development work.

(a) N. F. Swynnerton, E. P. McGovern, D. J. Mangold, J. A. Niño, E. M. Gause and L. Fleckenstein. HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma. Abstracts of Papers; Meeting of the Radiation Research Society, San Antonio, Texas, March 1983.

(b) N. F. Swynnerton, E. P. McGovern, D. J. Mangold, J. A. Niño, E. M. Gause and L. Fleckenstein. HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma. J. of Liq. Chromatogr., 6:1523-1534, 1983.

(c) N. F. Swynnerton, E. P. McGovern, J. A. Niño, and D. J. Mangold. An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma. Abstracts of Papers, Chemical Modifiers of Cancer Treatment Meeting, Banff, Alberta, Canada, November, 1983.

(d) N. F. Swynnerton, E. P. McGovern, J. A. Niño, and D. J. Mangold. An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma. Int. J. Radiation Oncology Biol. Phys., Vol. 10, pp. 1521-1524.

The initial HPLC method developed as described in papers (a) and (b) above and in Interim Report No. 2 (Appendix A) used ^{14}C-labeled ethiofos as internal standard. Later a more sensitive and selective method was developed [papers (c) and (d) above and Interim Report No. 3 (Appendix C)] which used WR 80855 as internal standard.

Copies of the above papers and reports which present the details of the methods which were used for the ethiofos plasma assays are included in Appendices A, B, C and D.

a. Development of a Non-radiolabeled Internal Standard for the Ethiofos (WR 2721) Plasma Assay

(1) Preliminary Evaluation

An HPLC system comprised of a Waters Associates Radial Compression Module (RCM-100) fitted with a C-18 cartridge and a mobile

phase of acetonitrile/water, (22:78) 0.01 M in tetrabutylammonium phosphate (TBAP) was found to widely separate the fluorescamine derivative of ethiofos from interferences in beagle plasma. After the elution of ethiofos a large "gap" existed in the chromatogram such that an appropriately-chosen material could be added to the sample, be resolved by the column and serve as an internal standard for the ethiofos assay.

Several of the homologs of ethiofos which were found to be unsuitable as internal standards in the previously-used HPLC separation system were evaluated with the current TBAP method. WR 80855 [S-3-(4-aminobutylamino)propyl phosphorothioate] was found to elute immediately after ethiofos and to be baseline separated from its homolog.

A preliminary stability study was performed on plasma solutions spiked with both ethiofos and WR 80855 to detect possible decomposition during storage over long periods of time. A fresh solution of ethiofos and WR 80855 was prepared to contain approximately 1 mg/mL of each. A 100-µL aliquot of this solution was added to 900 µL of thawed plasma to give a final component concentration of 100 ng/µL of each component. Portions (90 µL) of the plasma solutions were added to separate centrifuge tubes and placed in a freezer for storage (-20°C). Duplicate samples were removed and analyzed weekly over a period of four weeks.

For analysis, samples were allowed to thaw at room temperature prior to addition of C&L buffer, pH 7.6, followed by immediate derivatization with 400 µL of fluorescamine (5 µg/mL). After centrifuging, samples were then analyzed by HPLC using the following conditions:

Column:	Waters RCM-100 fitted with a 5 µm C-18 cartridge	
Mobile phase:	22% ACN 77% H_2O 1% TBAP (tetrabutylammoniumphosphate)	
Flow rate:	2 mL/min	
Filters:	395 nm excitation >460 nm emission	bandpass filters
Gain:	16X	

This preliminary stability study indicated that ethiofos decomposed at a slightly faster rate than WR 80855. Although both compounds tended to decompose in time, peak area ratios of the two showed that the compounds were fairly stable for a period of 2 weeks.

Results of the four-week study are found in Table 1 and Figure 1.

TABLE 1. ETHIOFOS AND WR 80855 STABILITY STUDY RESULTS

Time/Date	Day	Area Ethiofos	Area WR 80855	Ratio Ethiofos/A 80855
T_0/2-4	0	1270	644	1.97
		1320	690	1.91
T_1/2-7	3	1185	637	1.86
		1176	634	1.85
		1208	660	1.83
T_2/2-14	10	110*	58*	1.89
		1050	561	1.87
T_3/2-21	17	1529	884	1.73
		1500	878	1.71
$\bar{x}$		1280	699	1.85
SD		165	118	0.08
CV %		12.87	16.94	4.47

* - represents peak height in mm

$\bar{x}$ - mean

SD - standard deviation

CV % - relative standard deviation

Linear Regression Data from Table 1.

slope = -.00586 day^{-1}

y intercept = 1.93

correlation coefficient = -.869

at X = 21 days y' = 1.68

y' = Ratio Area ethiofos/Area 80855

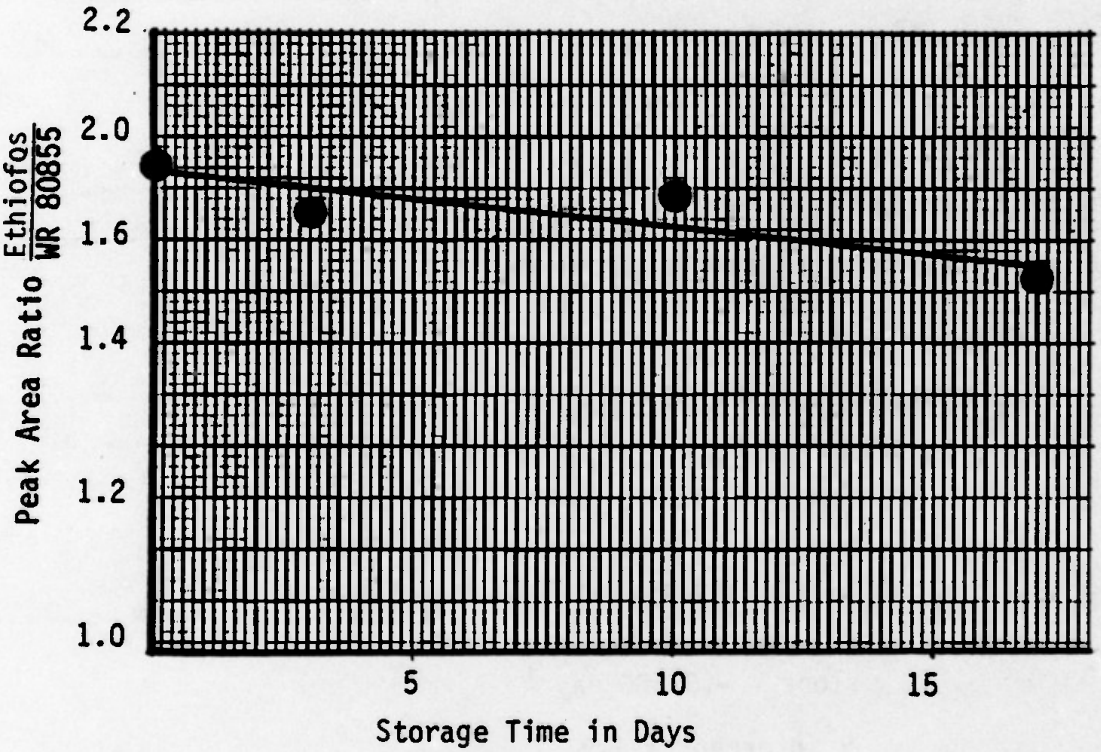


Figure 1. Relative Stability of Ethiofos and WR 80855 in Frozen Beagle Plasma, Average of Duplicate HPLC Analysis. Preliminary Study with Plasma Spiked with 100 µg Ethiofos per mL of Plasma.

An experiment was also performed to evaluate the stabilities of the fluorescamine derivatives of ethiofos and WR 80855. A 100-µL aliquot of a standard solution of ethiofos and WR 80855, 100 ng/µL each, was added to 200 µL of C&L borate buffer pH 7.6. The solution was derivatized by addition of 2 X 200-µL portions of fluorescamine reagent and the resulting solution was then injected repeatedly onto the HPLC column over a period of 65 minutes. The solution was stored in an ice/water bath between injections. Peak areas varied less than 3.3% over this time period and the area ratio of the ethiofos peak to the WR 80855 peak remained constant at 1.38 $\pm$ 0.04. This implied that the fluorescamine derivatives were stable in buffer for at least one hour at approximately 0°C. The raw data of this study are reported in Table 2 and Figure 2.

A similar experiment was performed to test the stability of the derivatives in plasma. A 90-µL portion of plasma and 100 µL of standard solution were added to 110 µL of C L borate buffer, pH 7.6. The solution was derivatized and analyzed as above. Total analysis time for five plasma samples was 265 minutes (4 hours, 25 minutes). The analysis time for plasma samples was longer because the column was flushed with a 90:10 acetonitrile water mobile phase after each injection to elute residual material left on the column. Once the column was thoroughly flushed, it was re-equilibrated. The fluorescamine derivatives of ethiofos and WR 80855 were found to be stable in plasma for a least one hour after derivatization when stored in an ice/water bath. Results of the plasma study are reported in Table 3 and Figure 3.

(2) Standard Calibration Curves

Various concentrations of ethiofos and WR 80855 were analyzed to collect relative response data for each component.

A stock solution containing both ethiofos (1 mg/mL) and WR 80855 (2 mg/mL) was prepared in C&L borate buffer, pH 10. Sequential 1:10 and 1:100 dilutions were performed on the stock solution and further dilutions gave ethiofos concentrations of 5, 10, 50, 100, 250 and 500 ng/µL. WR 80855 concentrations were twice those of ethiofos, i.e., 10, 20, 100, 200, 500, and 1000 ng/µL respectively. A calibration curve was constructed for each component (Figure 4) which indicated a linear response for both compounds over their respective concentration ranges.

(3) Characterization of (Ethiofos) WR 2721 Assay with WR 80855 as Internal Standard

(a) Detector Response and Calibration

Determination of detector response and generation of standard curves were carried out using HPLC system A, described in the Experimental Section. Sensitivity of the detector used for this work was about twenty times greater than that of the model previously used.

TABLE 2. RESULTS OF STABILITY STUDY OF ETHIOFOS AND WR 80855 FLUORESCAMINE DERIVATIVES IN BUFFER SOLUTION AT 0°C.

Time (min)	Area Ethiofos	Area WR 80855	Ratio Ethiofos/WR 80855
0	1980	1390	1.42
21	1989	1440	1.38
45	1913	1368	1.40
65	1853	1400	1.32
$\bar{x}$	1933	1400	1.38
SD	63.6	30.1	0.04
CV %	3.29	2.15	3.13

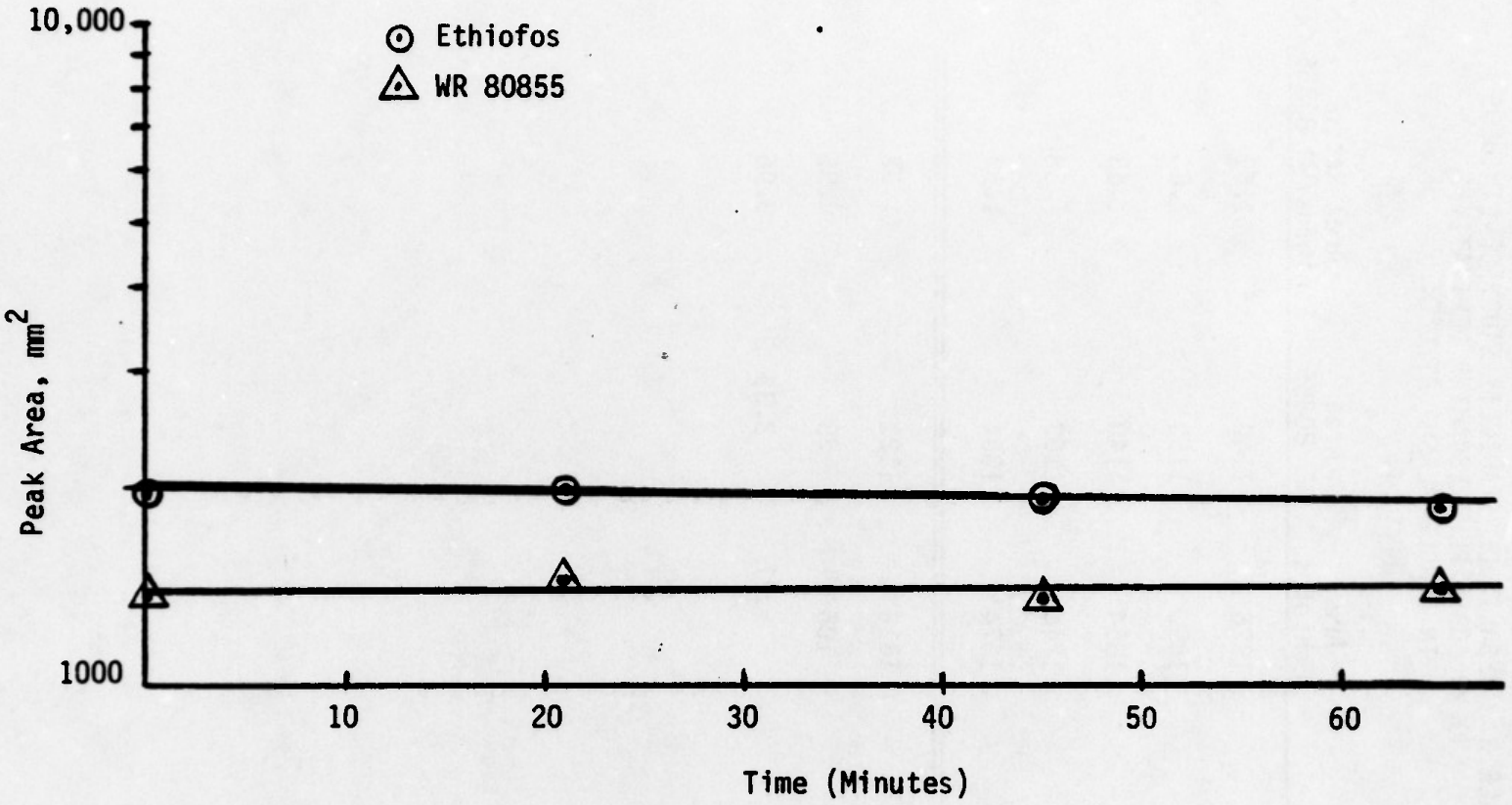


Figure 2. Stabilities of Ethiofos and WR 80855 Fluorescamine Derivatives in Buffer Solution at ~0°C.

TABLE 3. RESULTS OF STABILITY STUDY OF ETHIOFOS AND WR 80855 FLUORESCAMINE DERIVATIVES IN BEAGLE PLASMA AT 0°C.

Time (min)	Area Ethiofos	Area WR 80855	Area Ratio Ethiofos/WR 80855
0	1926	1140	1.69
65	1890	1159	1.63
105	1854	1140	1.63
212	1746	1102	1.58
255	1674	1064	1.57
$\bar{x}$	1818	1121	1.62
SD	105	38	0.05
CV %	5.77	3.39	2.96

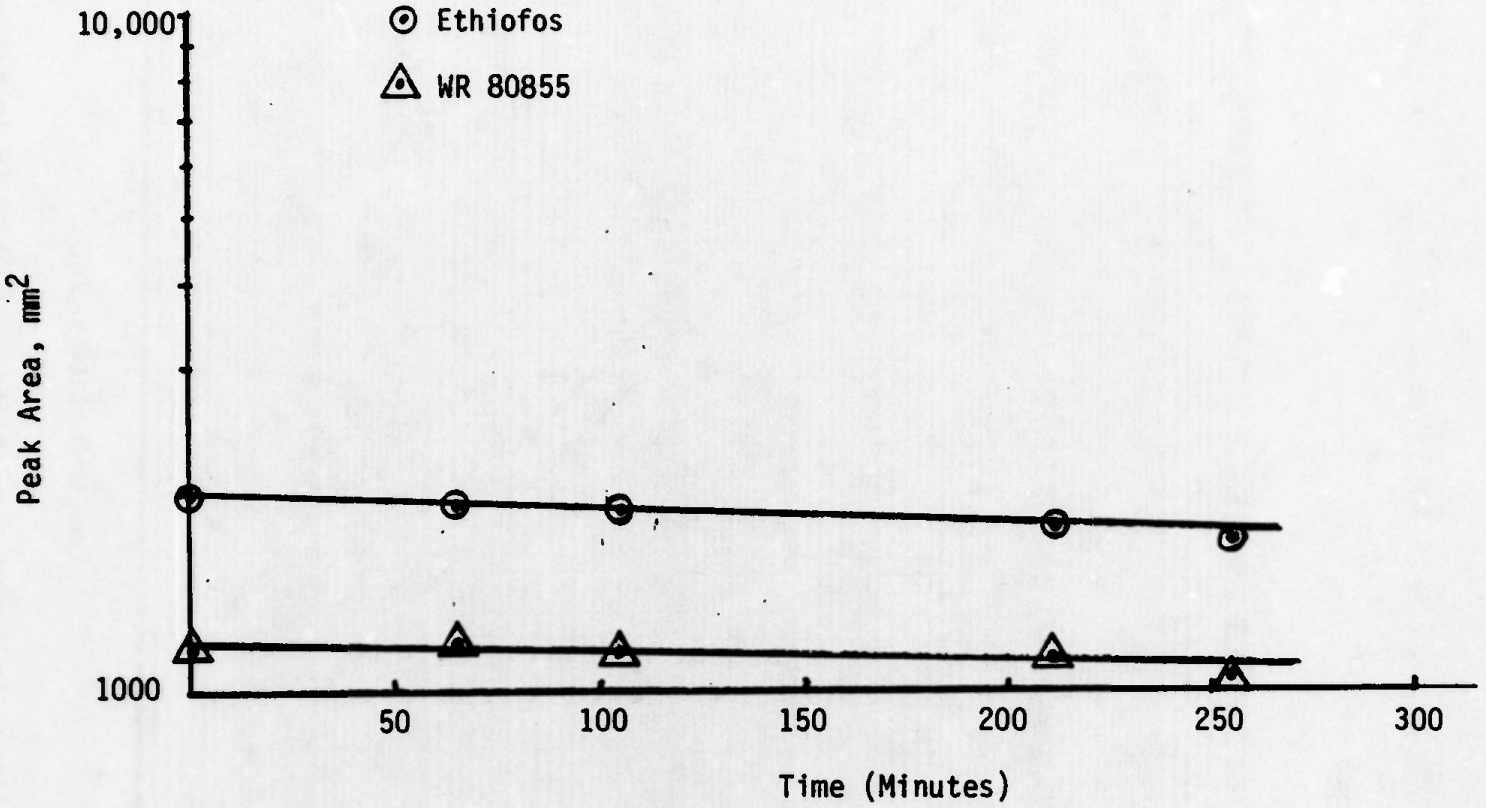


Figure 3. Stability of Ethiofos and WR 80855 Fluorescamine Derivatives in Plasma at ~0°C.

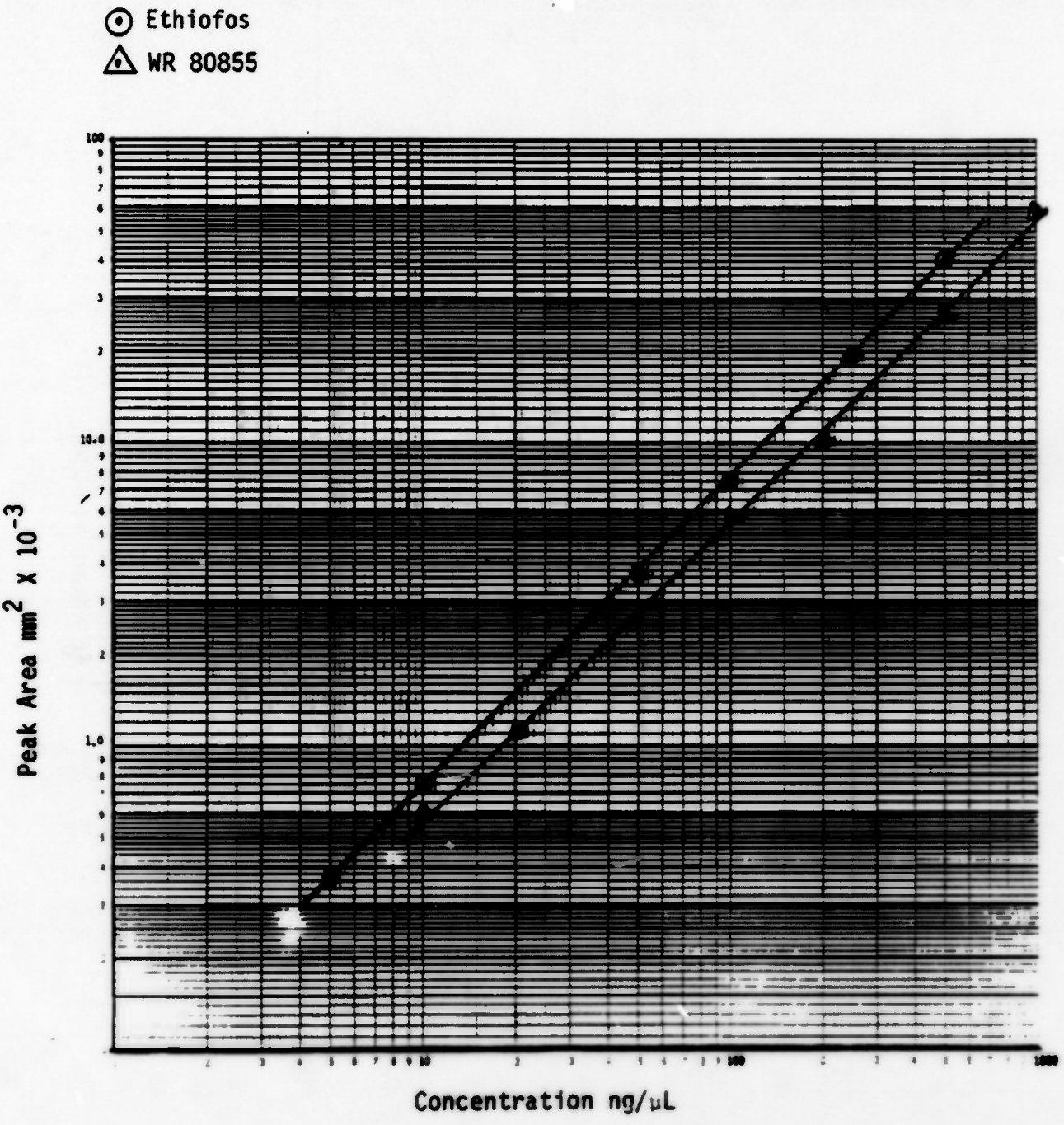


Figure 4. Calibration Curves for Ethiofos and WR 80855 in Buffer Solution.

Three calibration curves were constructed using solutions of ethiofos and WR 80855 in plasma and in pH 10 borate buffer to span the concentration range of 1000 µg/mL to near the minimum detectable limit. Curve A spanned the range 0.25 to 10.0 µg/mL and each calibration sample contained 22.2 µg/mL of WR 80855. Curve B spanned the range 10.0 to 100 µg/mL and 222 µg/mL WR 80855 was used as internal standard. Curve C spanned the highest range of 100 to 1000 µg/mL and 1110 µg/mL of WR 80855 was contained in each calibration sample.

All samples were freshly made and individually derivatized according to the normal procedure then immediately injected onto the HPLC column. Plots of the peak area ratio of ethiofos to WR 80855 versus ethiofos concentration gave excellent straight-line fits for each of the curves. The raw data used to construct the curves along with the regression results are presented in Tables 4 and 5, and the calibration curves are presented in Figures 5 through 12. As can be seen from a statistical analysis of the response of WR 80855, there is fairly good repeatability at each of the three concentration levels. Considering that six volumetric measurements must be made in the preparation of each sample in addition to the injection volume measurement and the integrator repeatability, the overall repeatability is excellent. The high values obtained for the linear regression correlations (R^2) reflect the smoothing effect of using an internal standard.

Comparison of the two sets of curves (ethiofos in buffer versus ethiofos in plasma) revealed essentially the same response in either medium, within the error of the method. The curve of low level ethiofos in plasma showed a positive Y-intercept which was due to a small interference present in the beagle plasma. The interference corresponded to an ethiofos concentration of about 0.3 µg/mL.

(b) Column Variability

Although flushing the analytical column after each injection maintained column efficiency for a reasonable lifetime (usually greater than 100 injections), band spreading and a decrease in resolution ultimately occurred and necessitated renewing the cartridge. Once when it became necessary to replace a cartridge which had developed a leak after more than 100 injections, a repeat of the last analysis on the old column was performed. The results are summarized below. Close correspondence of the peak ratios implied that the system was insensitive to moderate losses in resolution between ethiofos and WR 80855 and hence to change of analytical columns.

Sample Identity	Conditions	Peak Area		Peak Ratio
		Ethiofos	WR 80855	Ethiofos/WR 80855
Ethiofos 3.0 µg/mL standard	Old Column	112259	496254	0.22609
"	New Column	104518	448208	0.23319

TABLE 4. CALIBRATION CURVE DATA--ETHIOFOS IN BUFFER SOLUTION, WR 80855 AS INTERNAL STANDARD

Calibration Curve A--0.25 to 10.0 µg/mL

Ethiofos µg/mL	Peak Area, integrator units[a] Ethiofos	WR 80855[b]	Area Ratio Ethiofos/WR 80855
0	0	-	0
0.25	9152	490569	0.01866
0.50	17300	481229	0.03595
1.00	30624	390063	0.07851
3.00	112259	496524	0.22609
3.00	104518	448208	0.23319
5.00	172001	463284	0.37126
7.50	244760	436744	0.56042
10.0	344819	442167	0.77984

$\bar{x}$: 456,098 slope: 0.0768
s: 34,841 intercept: -0.0021
CV: 7.6% R^2: 0.9995

Calibration Curve B--10.0 to 100 µg/mL

Ethiofos µg/mL	Peak Area, integrator units[c] Ethiofos	WR 80855[d]	Area Ratio Ethiofos/WR 80855
0	0	-	0
10.0	3341	41028	0.08143
25.0	8252	40557	0.20347
50.0	17622	43999	0.40051
75.0	27050	45404	0.59576
100.0	33798	43083	0.78449

$\bar{x}$: 42,814 slope: 0.0785
s: 2,029 intercept: 0.00402
CV: 4.7% R^2: 0.9999

Calibration Curve C--100 to 1000 µg/mL

Ethiofos µg/mL	Peak Area, integrator units[c] Ethiofos	WR 80855[e]	Area Ratio Ethiofos/WR 80855
0	0	-	0
100	25378	155257	0.16346
250	75246	188762	0.39863
500	161474	195346	0.82661
750	253254	212570	1.19139
1000	327710	205260	1.59656

$\bar{x}$: 191,439 slope: 0.0159
s: 22,187 intercept: 0.00495
CV: 11.6% R^2: 0.9998

a Detector range setting of 1
b 22.2 µg/mL
c Detector range setting of 100 (attenuation factor)
d 222 µg/mL
e 1110 µg/mL

TABLE 5. CALIBRATION CURVE DATA--ETHIOFOS IN BEAGLE PLASMA, WR 80855 AS INTERNAL STANDARD

Calibration Curve A--0.25 to 10.0 µg/mL

Ethiofos µg/mL	Peak Area, integrator units[a] Ethiofos	WR 80855[b]	Area Ratio Ethiofos/WR 80855
0	10631	284890	0.03732
0.25	19644	470600	0.04174
0.50	21194	455931	0.04649
1.00	35536	478039	0.07434
3.00	169551	515874	0.32867
6.00	212188	411852	0.51520
10.0	449621	523254	0.85928

$\bar{x}$: 448,634 slope: 0.0840
s: 81,297 intercept: 0.0235
CV: 18.1% R^2: 0.9964

Calibration Curve B--10.0 to 100 µg/mL

Ethiofos µg/mL	Peak Area, integrator units[c] Ethiofos	WR 80855[d]	Area Ratio Ethiofos/WR 80855
10.0	4132	46538	0.08879
25.0	9832	43781	0.22457
50.0	18798	43708	0.43008
75.0	27595	43771	0.63044
100.0	36671	44600	0.82222
10.0	3768	41302	0.09145

$\bar{x}$: 43,950 slope: 0.00816
s: 1,688 intercept: 0.0141
CV: 3.8% R^2: 0.9997

Calibration Curve C--100 to 1000 µg/mL

Ethiofos µg/mL	Peak Area, integrator units[c] Ethiofos	WR 80855[e]	Area Ratio Ethiofos/WR 80855
100	33489	188892	0.1773
250	84766	203554	0.4165
500	179548	220734	0.8134
750	273648	236200	1.1586
750	278883	234161	1.1910
1000	331944	205670	1.6140
1000	299849	190517	1.5739

$\bar{x}$: 211,388 slope: 0.001563
s: 19,397 intercept: 0.0206
CV: 9.2% R^2: 0.9994

a Detector range setting of 1
b 22.2 µg/mL
c Detector range setting of 100 (attenuation factor)
d 222 µg/mL
e 1110 µg/mL

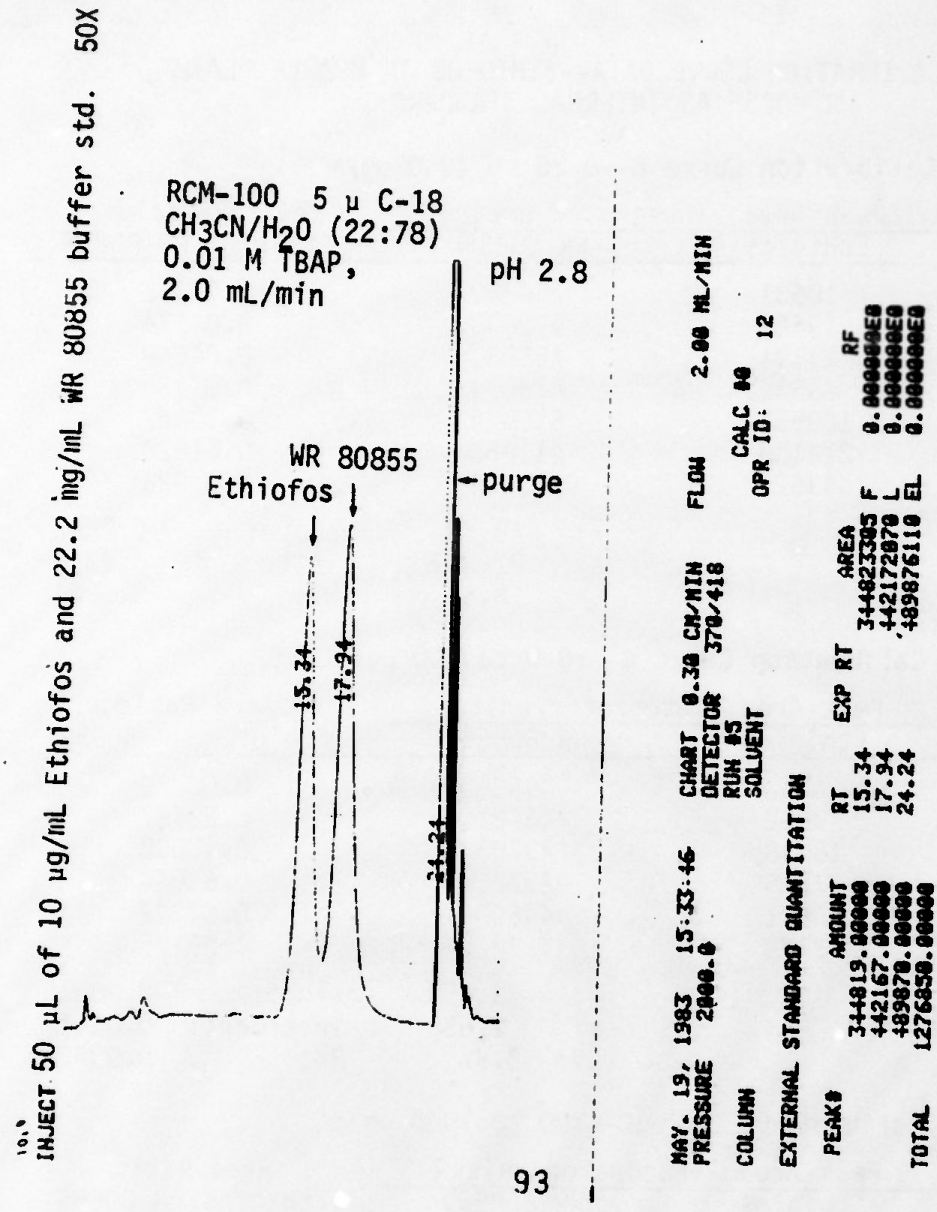


Figure 5. HPLC Chromatogram Showing Separation of Ethiofos (10 µg/mL) and WR 80855 (22.2 µg/mL) in pH 10 Borate Buffer.

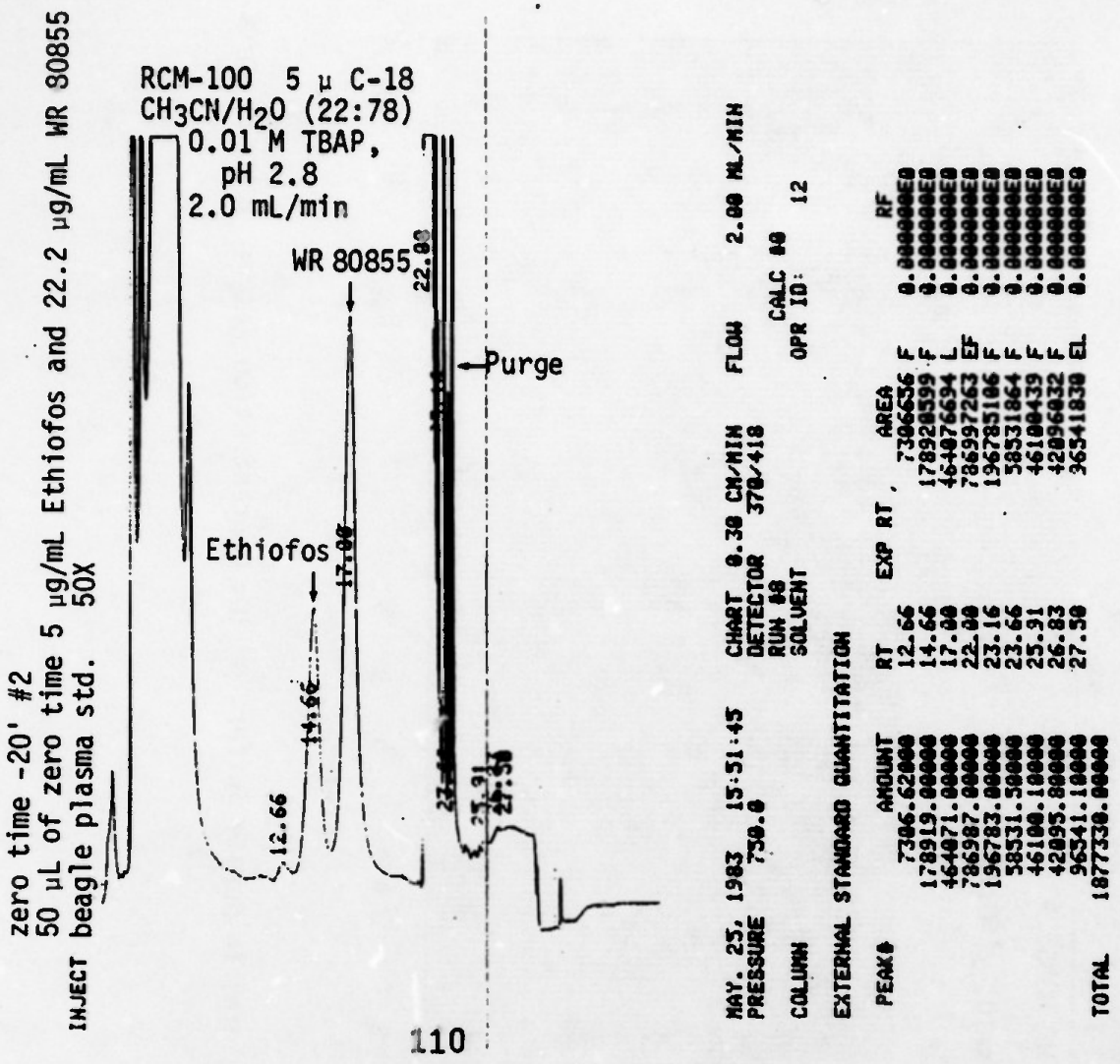


Figure 6. HPLC Chromatogram Showing Separation of Ethiofos (5 µg/mL) and WR 80855 (22.2 µg/mL) in Beagle Plasma.

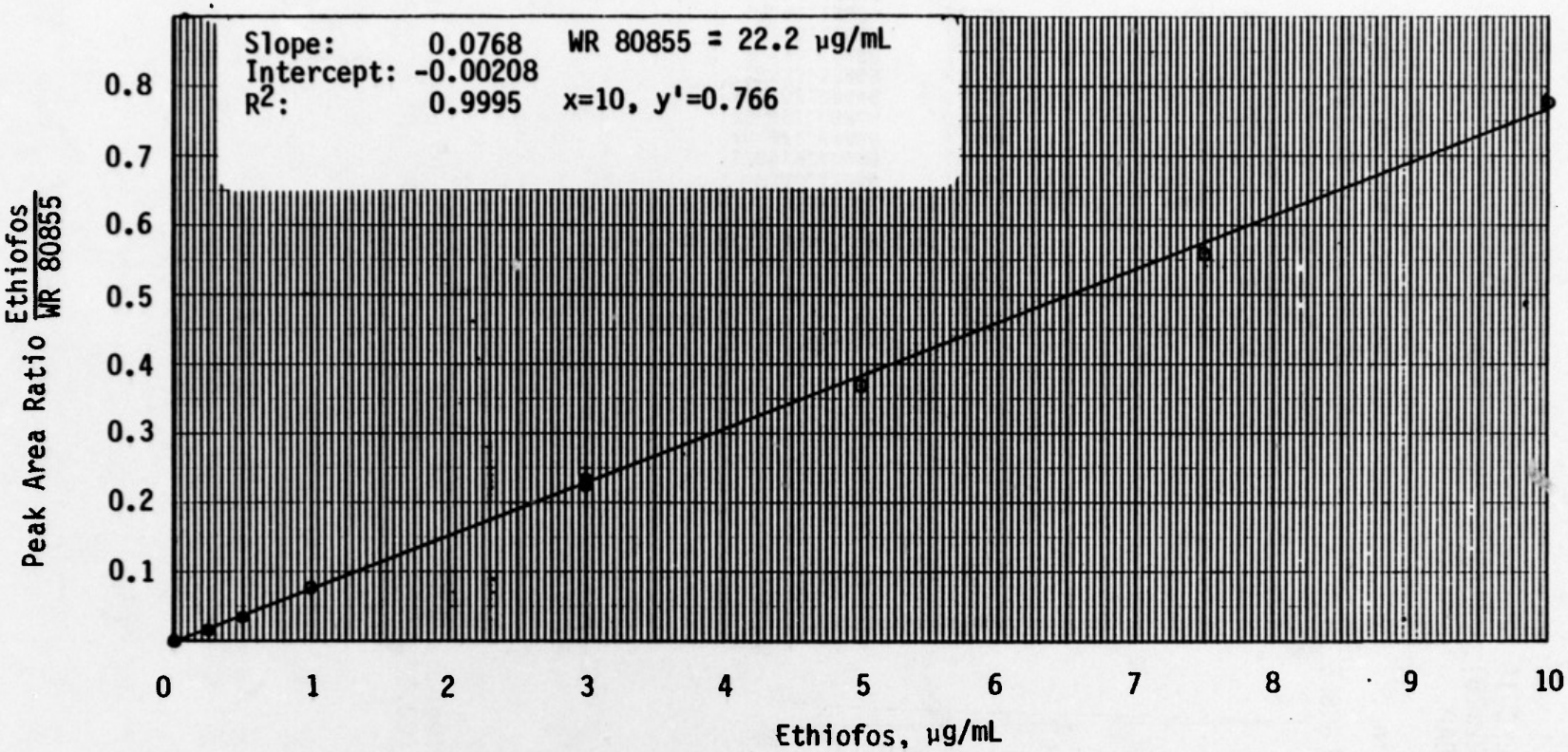


Figure 7. Calibration Curve A, Ethiofos in Borate Buffer, pH 10; Concentration Range: 0 to 10 µg/mL.

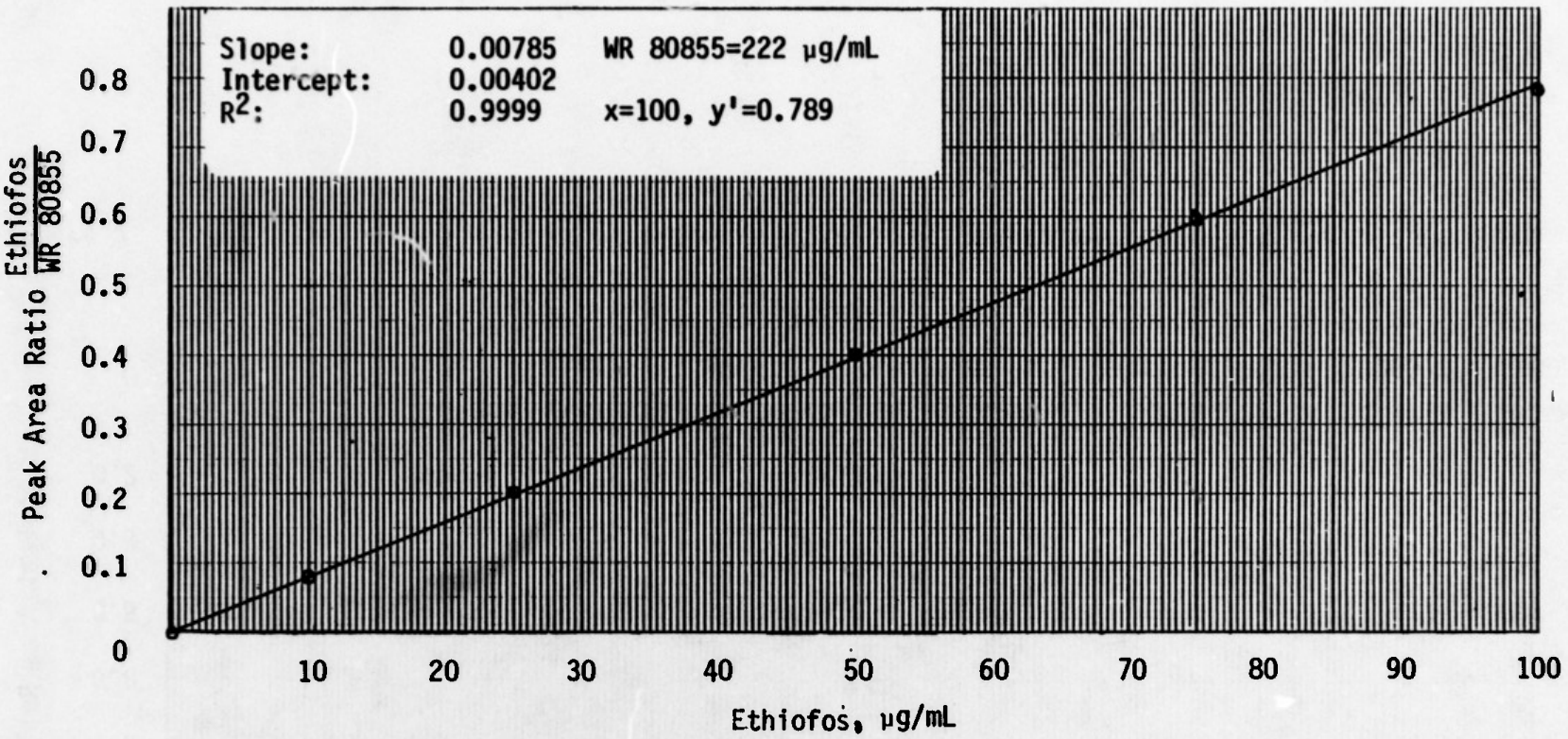


Figure 8. Calibration Curve B, Ethiofos in Borate Buffer, pH 10; Concentration Range: 0 to 100 µg/mL.

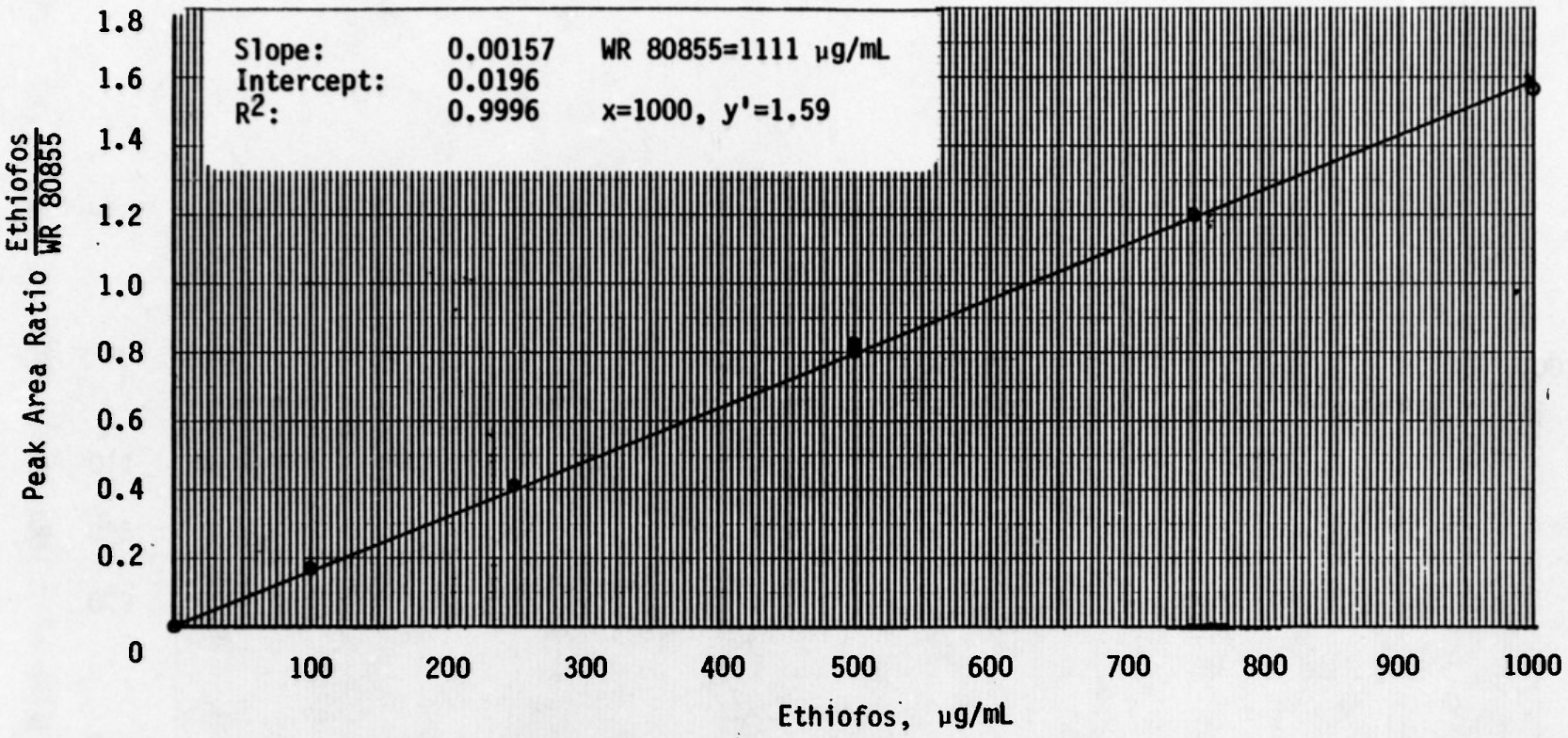


Figure 9. Calibration Curve C, Ethiofos in Borate Buffer, pH 10; Concentration Range: 0 to 1000 µg/mL.

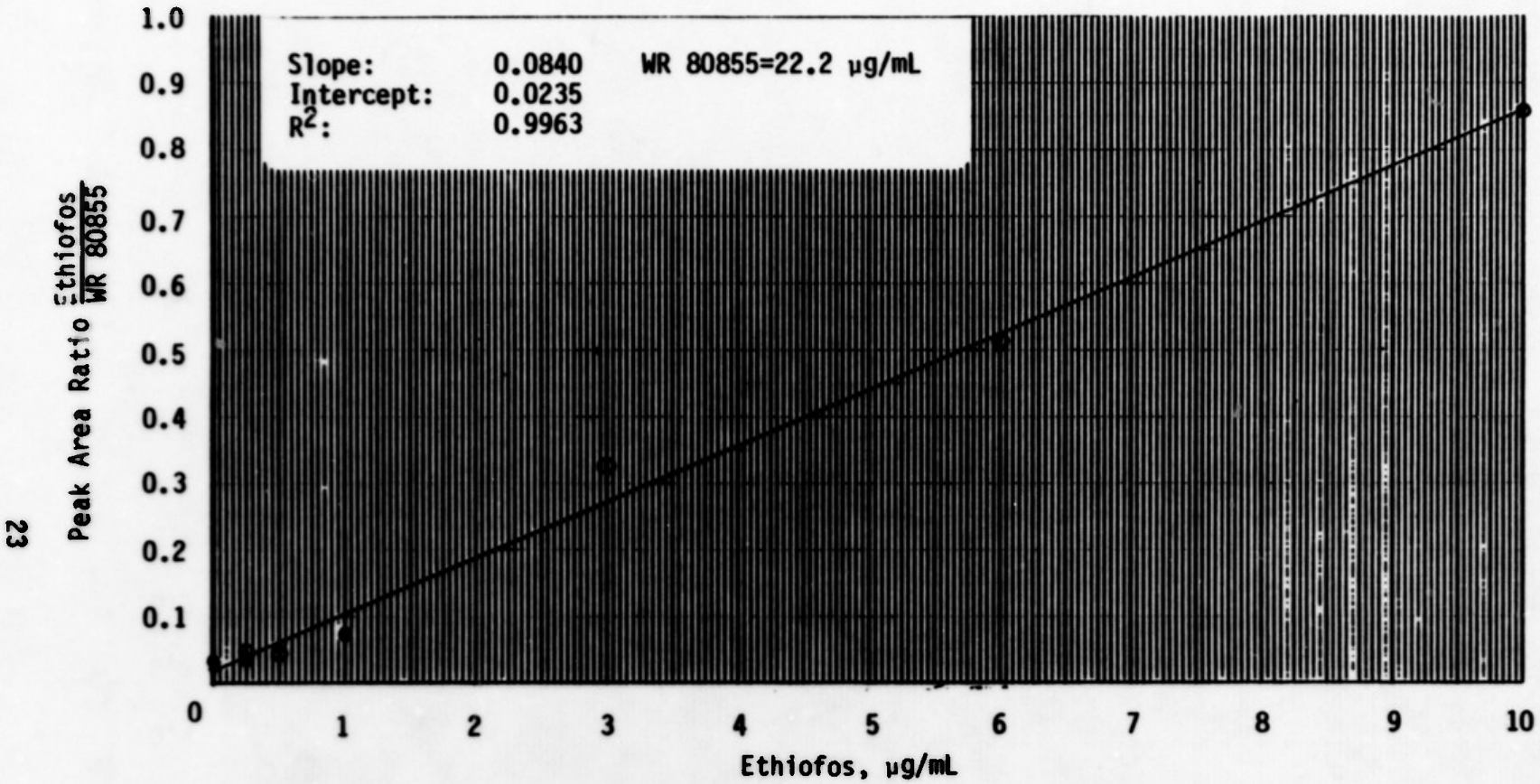


Figure 10. Calibration Curve A, Ethiofos in Beagle Plasma; Concentration Range: 0 to 10.0 µg/mL.

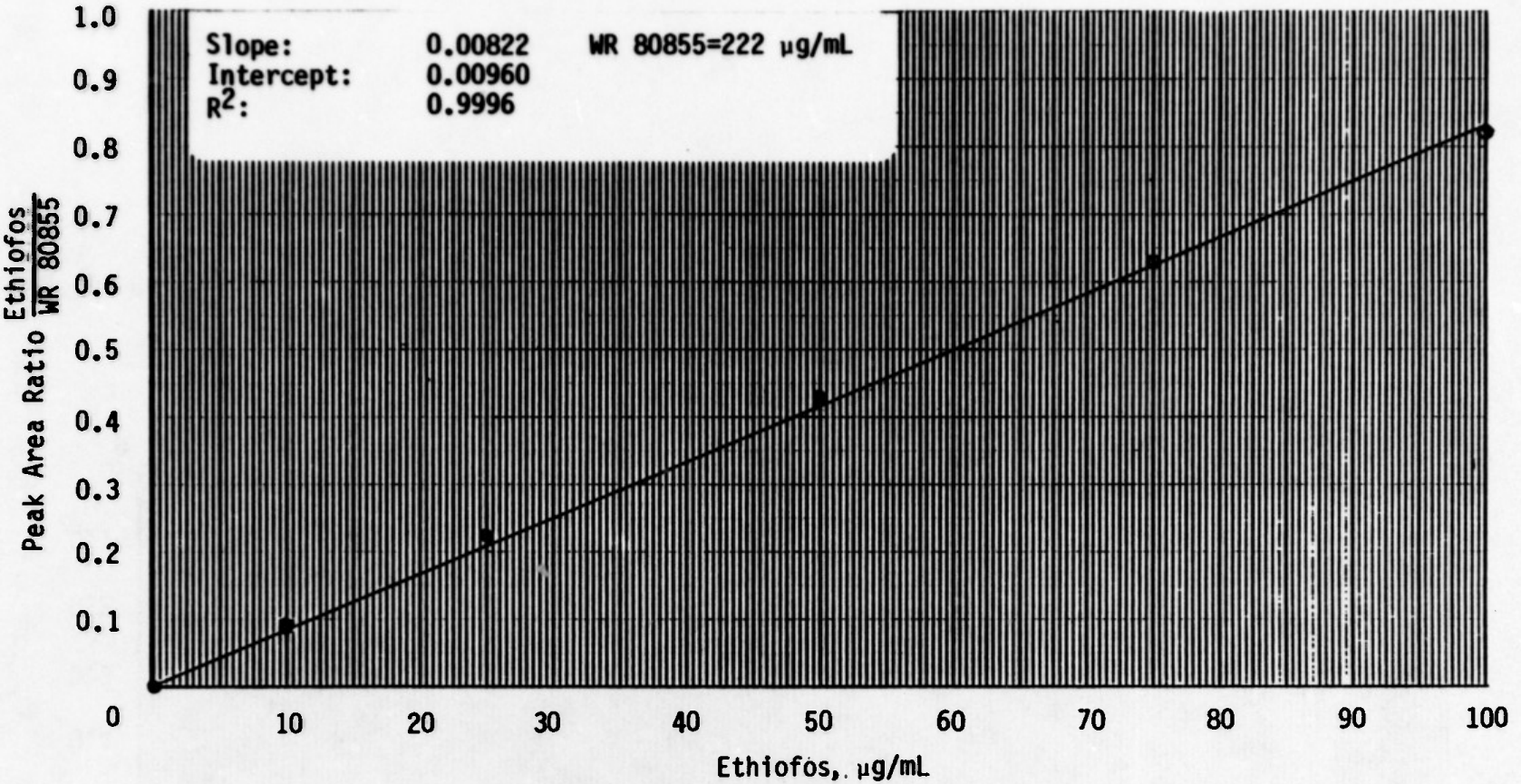


Figure 11. Calibration Curve B, Ethiofos in Beagle Plasma; Concentration Range: 0 to 100 µg/mL.

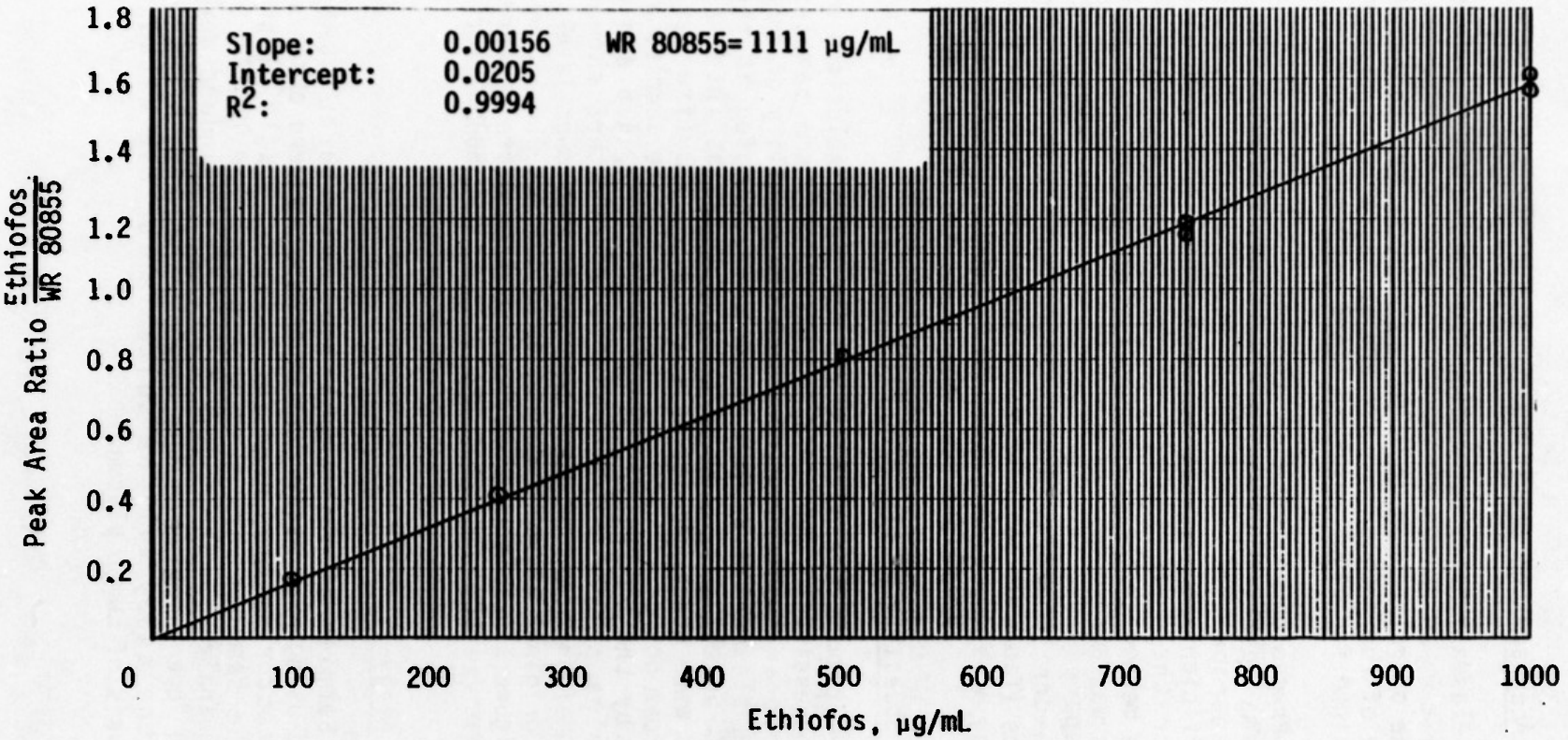


Figure 12. Calibration Curve C, Ethiofos in Beagle Plasma; Concentration Range: 0 to 1000 µg/mL.

(c) Accuracy and Precision

Plasma samples containing randomly-chosen levels of ethiofos within four concentration ranges (0-1.0, 1.0-10.0, 10.0-100, 100-1000) and spanning the overall range of 0.25 to 770 µg/mL were assayed to determine the accuracy of the method. An average deviation of 5.5% from the actual value was obtained for eight determinations (Table 6).

Precision estimates were obtained from replicate analyses of beagle plasma spiked with ethiofos at the 0.5, 5, 50, and 500 µg/mL levels. An appropriate amount of WR 80855 was added as an internal standard. Coefficients of variation ranged from 1.4 to 5.0% with an average of 2.9% for the four levels (Table 7). Extensive characterization of the method at the lower detection levels by replicate analysis of blind samples was also done. Eighteen blind samples at three concentrations were prepared by L. Fleckenstein of WRAIR. An additional six samples were independently prepared at a level near the minimum detectable limit. Results (Table 8) demonstrated that essentially the same precision and accuracy was attainable at the lowest levels of detectability.

(d) Stability of Biological Samples

Although the stability of buffered solutions of ethiofos is known[1], the necessity to store drug-containing plasma samples for long periods prior to analysis required that the stability of such samples be known. Beagle plasma was spiked to contain ethiofos at the 0.5-, 5.0-, 50- and 500-µg/mL levels, WR 80855 was added as internal standard, and the samples were quick frozen and stored at -20°C (freezer) and at -78°C (in a styrofoam box containing dry ice). Duplicate samples at each level were assayed by the described method after 1, 2, 4.5 and 6 months storage. Samples stored at the higher temperature showed appreciable decomposition after 2 months, particularly at the lower levels, with 50% or less of the original ethiofos remaining of the 0.5 and 5.0 µg/mL levels (Table 9 and Figure 13). Storage at dry ice temperature appeared to have completely preserved the ethiofos (relative to WR 80855) for 6 months.

(e) Assay of Human Plasma

Samples of plasma obtained from three human volunteers were assayed using HPLC assay. No interferences were observed at the most sensitive detector setting used for assay of beagle plasma (Figure 14a, b, c). The samples were also spiked with low levels of ethiofos and WR 80855 and analyzed. The drug and internal standard were observed to elute in an "open" region of the chromatogram (Figure 15a, b, c). It appears that the assay will be suitable for use without modification in the analysis of human plasma.

TABLE 6. ACCURACY OF ETHIOFOS PLASMA ASSAY

Spike Level, µg/mL	Measured Level, µg/mL	Percent Deviation (D)
0.25	0.27	8.0
0.75	0.72	-4.0
8.0	7.7	-3.8
15.0	16.4	9.3
40.0	36.3	-9.2
90.0	83.0	-7.8
320	315	-1.6
770	765	-0.6

Average Deviation = $\frac{|D|}{n}$ = 5.5%

TABLE 7. PRECISION ESTIMATES OF ETHIOFOS PLASMA ASSAY USING WR 80855 AS INTERNAL STANDARD[a]

Ethiofos Plasma Level, µg/mL	0.5	5.0	50	500
Number of Replicates	7	7	5	6
Average peak height ratio	0.576	0.258	0.516	1.06
Standard deviation	0.0206	0.0116	0.00734	0.018
CV, %	3.6	5.0	1.4	1.7

a. Mobile phase of CH_3CN/EtOH/water, 0.01 M in TBAP, 2.0 mL/min.

TABLE 8. ANALYSIS OF BLIND SAMPLES

	Assays Ethiofos Level, µg/mL			
	Level 1	Level 2	Level 3	Level 4
	0.104	0.51	1.1	6.9
	0.096	0.50	1.2	7.4
	0.096	0.51	1.2	7.4
	0.095	0.52	1.2	7.7
	0.087	0.51	1.2	7.6
	0.077	0.57	1.2	
Mean	0.092	0.520	1.18	7.40
Standard Deviation	0.00931	0.0253	0.0408	0.308
CV	10.1%	4.9%	3.4%	4.2%
True Value	0.090	0.54[a]	1.10[a]	7.50[a]
Recovery	102.8%	96.3%	107.0%	98.7%

a. Values of blind samples prepared and reported by L. Fleckenstein. A single blank was included which gave no value for ethiofos, demonstrating no interference.

TABLE 9. STABILITY OF ETHIOFOS IN STORED FROZEN PLASMA[a]

Temperature, °C	Ethifos Level, µg/mL									
	Zero Time[b]	(%)	1 Month[c]	(%)	2 Months[c]	(%)	4.5 Months[c]	(%)	6 Months	(%)
-20	0.50	(100)	0.39	(77)	0.17	(34)	0.08	(16)	0.10	(20)
-78	0.50	(100)	-		0.48	(96)	0.56	(112)	0.49	(98)
-20	5.0	(100)	3.9	(78)	2.5	(50)	1.2	(24)	0.94	(19)
-78	5.0	(100)	5.1	(102)	4.0[d]	(80)	4.7	(94)	4.52	(90)
-20	50	(100)	41	(82)	38	(76)	31	(62)	25.8	(52)
-78	50	(100)	50	(100)	48	(96)	49	(98)	55.6	(111)
-20	500	(100)	440	(88)	415	(83)	347	(69)	332	(66)
-78	500	(100)	485	(97)	500	(100)	523	(105)	548	(110)

a. Beagle plasma, spiked with ethiofos and internal standard, quick frozen and stored at -20°C or -78°C.

b. Calculated from added volume of freshly prepared standard solutions.

c. Values obtained as average of two determinations using HPLC method described herein.

d. Single determination.

○ , ● - Original concentration of 500 µg ethiofos per mL of beagle plasma

△ , ▲ - Original concentration of 50 µg ethiofos per mL of beagle plasma

□ , ■ - Original concentration of 5.0 µg ethiofos per mL of beagle plasma

○ , ● - Original concentration of 5.0 µg ethiofos per mL of beagle plasma

Open symbols ⟶ samples stored at -78°C
Closed symbols ⟶ samples stored at -20°C

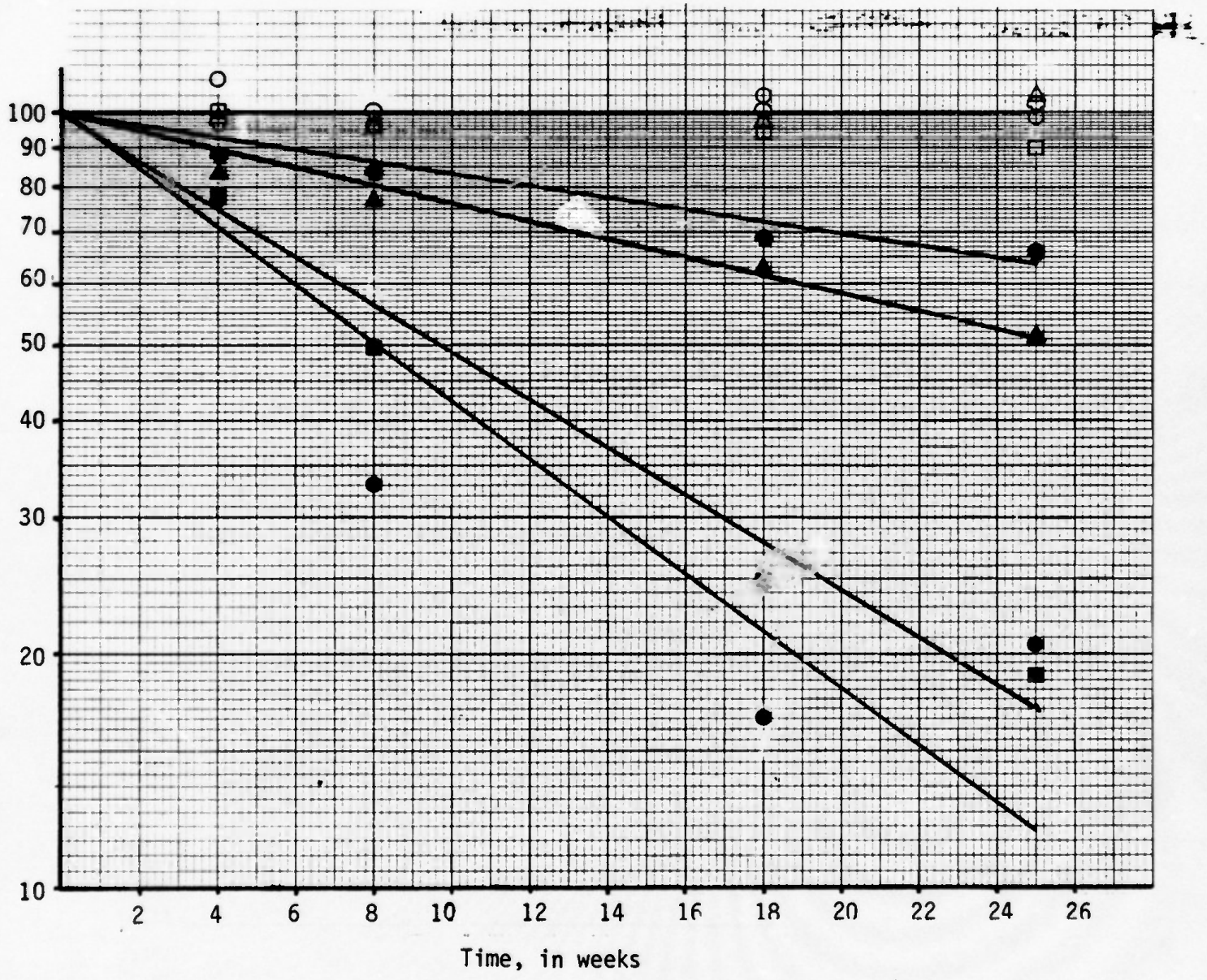


Figure 13. Time Course of Ethiofos in Frozen Plasma at -20° and at -78°C.

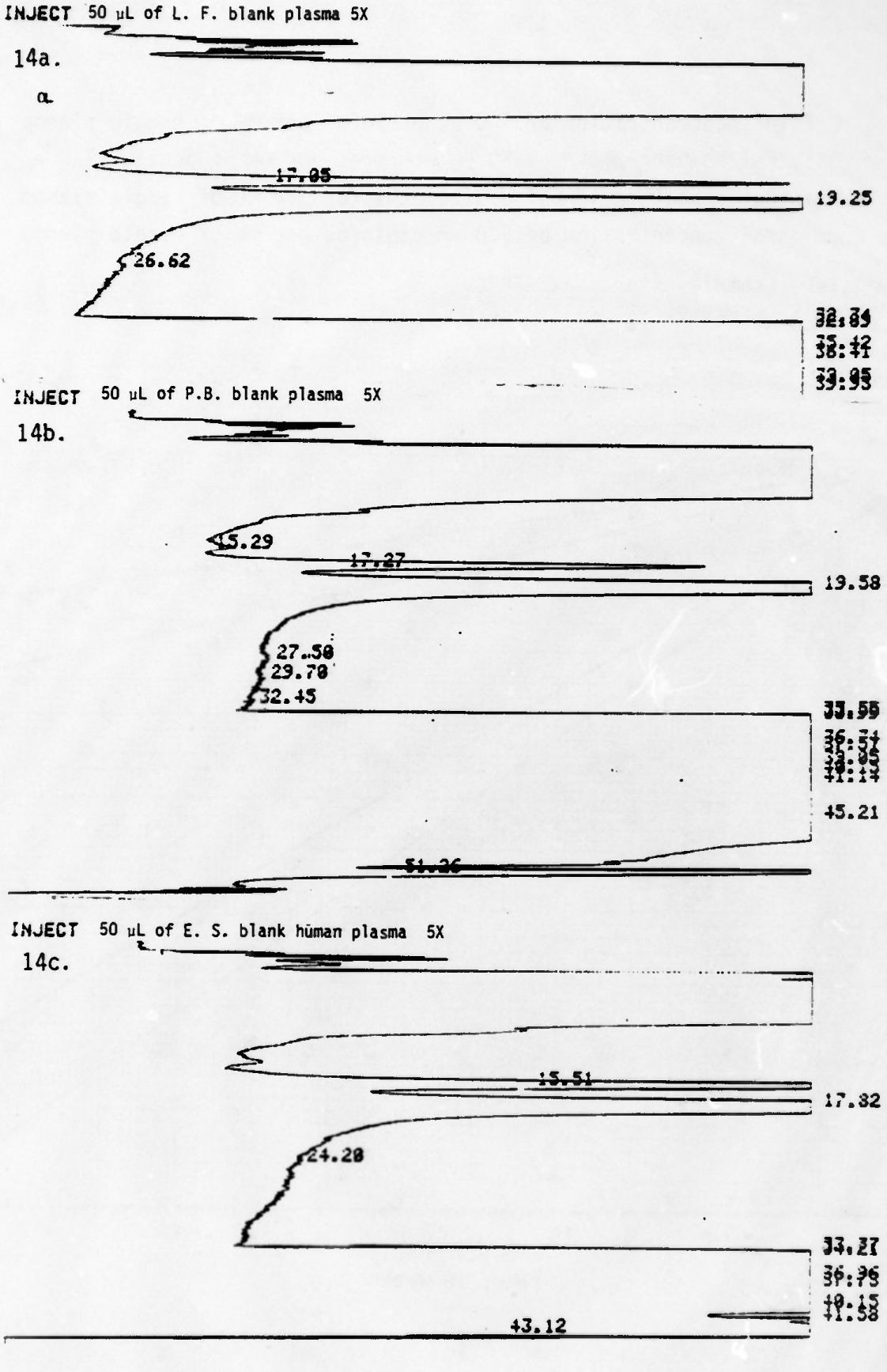


Figure 14a,b,c. Chromatograms of Derivatized Human Plasma Samples.

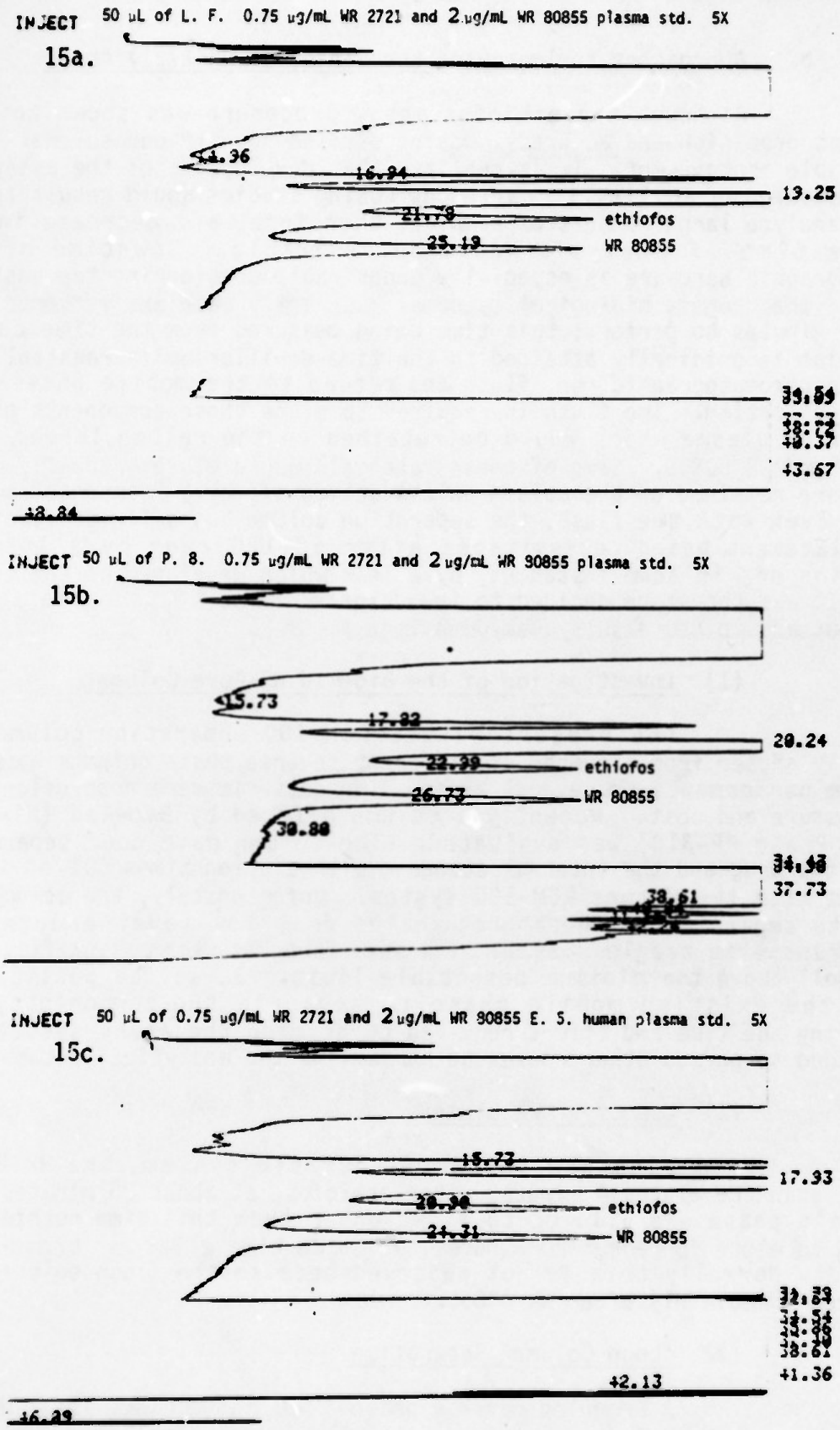


Figure 15a,b,c. Chromatograms of Human Plasma Samples Spiked with Ethiofos and WR 80855 and Derivatized.

b. Approaches to Improving the Ethiofos (WR 2721) Assay

Although the ethiofos assay procedure was shown to have excellent precision and accuracy, dosing studies pointed out several areas for possible improvement. It is realized that development of the assay and the accompanying ability to carry out dosing studies would result in the need to analyze large numbers of samples; therefore, any decrease in the average time of analysis would be desirable. Downtime of the chromatographic hardware is especially undesirable considering the unstable nature of the drug in biological systems. Currently each analysis requires about 50 minutes to perform; this time being measured from the time column equilibrium is originally attained to the time equilibrium is reestablished after the chromatographic run, flush and return to the mobile phase used for the separation. The flush is required to elute those components of the derivatized plasma which would be retained on the column longer than ethiofos and WR 80855. Some of these materials would elute gradually while others were retained on the column unless driven off by a "stronger" mobile phase. Even with the flush, the separation column has a finite lifetime, its replacement being necessitated after 60-120 runs by a loss in resolution or, in some instances, by a leak which developed at the column inlet. It was therefore decided to investigate means to increase sample throughput and to decrease system downtime.

(1) Investigation of the Bio-Rad Hi-Pore Column

The present Waters RCM-100 separation column was originally chosen from a number of different reverse phase columns based on superior performance in several areas. The criteria were resolution, low back pressure and cost. Recently a column offered by Bio-Rad (Hi-Pore Reverse Phase RP-318) was evaluated. The column gave good separation between the drug and the internal standard with elution times 60% of those obtained with the Waters RCM-100 system. Unfortunately, the column was unable to separate the phosphorothioates from low-level endogenous interferences in beagle plasma. The interferences became significant at levels well above the minimum detectable limit. It may be possible to modify the existing mobile phase to separate the components, but considering the time and effort required to develop the assay system, it was decided to pursue other routes to streamline the analytical method.

(2) Use of a Precolumn

With the present isocratic system, the WR 80855 internal standard elutes 4 minutes after ethiofos, at about 26 minutes. If the mobile phase was allowed to flow longer than this time nothing was observed to elute for about 20 minutes, at which time a large, broad peak appeared. Normally this is not observed because the flush solution is introduced immediately after WR 80855.

(3) "Loop Column" Separation

A Brownlee reverse phase loop column was attached to the Rheodyne Injector in such a way that the entire sample was forced to

pass onto the column immediately after injection but the column could be isolated from the system at any time by operating a valve. This is a procedure often used in gas chromatography but infrequently in HPLC. In theory, if the desired materials could be separated from the later-eluting impurities using the small loop column, the impurities could be backflushed to waste while the compounds of interest were undergoing separation in the larger analytical column. The efficiency of the small column was not great enough to perform this function, for peaks of interest and impurities were eluted together or were not eluted at all, depending upon when the valve was operated. This technique could be made to work with a precolumn capable of sufficient resolution. Certainly, a column such as the RCM-100 would be capable of performing this function, but at a cost near $2000 per unit and with the loss of system simplicity.

(4) Sample Treatment Prior to Injection

Preinjection sample treatment was investigated using two kinds of commercially available columns. Plasma samples spiked with the drug and with internal standard were passed through reverse phase Sep Pak® columns and the columns were flushed with small amounts of solvent. In a typical experiment, 0.5 mL of spiked plasma was forced through a column which had been "activated" by treating first with acetonitrile/water and with water. The plasma was flushed through with 1.0 mL of pH 10 borate buffer and the column effluent was analyzed in the usual way. Up to 90% of the material eluting after WR 80855 was removed in the best experiments. Although this was encouraging, the remaining 10% which was not removed was sufficient to require that the flush procedure of the assay be retained. Quantitative separation of the phosphorothioates from the impurities was not achieved in any of the experiments.

(5) Extraction of Plasma

Beagle plasma spiked with both ethiofos and WR 80855 was extracted with the following organic solvents in attempts to "clean up" the samples; ethyl ether, dichloromethane, chloroform and ethyl acetate. The extents to which the lipid-like materials were removed was not immediately apparent in the HPLC analyses because the materials which would be expected to be removed by this process probably would not elute from the reverse phase column before the flush was begun. It was assumed that nonpolar materials were removed by the extractions and that this could be of some benefit to the HPLC system. A 50% increase in sensitivity was observed which was ascribed to partitioning of acetone (solvent for fluorescamine) into the organic phase resulting in a concentration of the water soluble fluorescamine derivatives of ethiofos and WR 80855.

2. WR 1065

During the period covered by this annual report an HPLC method for the analysis of WR 1065 in plasma has been developed and the following presentation at a technical meeting and paper resulted.

- E. P. McGovern, N. F. Swynnerton, P. D. Steele and D. J. Mangold. HPLC Assay for 2-(3-Aminopropylamino)ethanethiol (WR 1065) in Plasma. Abstracts of Papers, Chemical Modifiers of Cancer Treatment Meeting, Banff, Alberta, Canada, November, 1983.
- E. P. McGovern, N. F. Swynnerton, P. D. Steele and D. J. Mangold. HPLC Assay for 2-3-(Aminopropylamino)ethanethiol (WR 1065) in Plasma. Int. J. Radiation Oncology Biol. Phys., Vol. 10, pp. 1517-1520.

The procedure as presented in the above papers was used for the WR 1065 plasma assays as discussed later. Copies of the papers are attached as Appendix E.

Subsequent to the Banff conference mentioned above, a series of studies were initiated to elucidate the stability of WR 1065 and WR 1729, the compound used as an internal standard, in both plasma and other solutions. The results obtained are described below. The results of analysis of the plasma samples for WR 1065 from the ethiofos dog dosing No. 8 (12/14/83) are presented under Section IV.B.3., In Vivo Studies.

a. Stability Studies in Plasma and 1:1 0.1 M Monochloroacetic Acid (MCAA)/0.2 M Perchloric Acid ($HClO_4$) at 4°C

Both canine plasma (3.0 mL) and mixed 1:1 0.1 M MCAA/0.2 M $HClO_4$ at 4°C were spiked with 30 µL of a 1:1, 0.1 M MCAA/0.2 M $HClO_4$ solution containing 5 and 10 µg/µL of WR 1065 and WR 1729 respectively. Both samples were vortexed briefly after fortification and replaced in an ice bath. Portions (100 µL) were withdrawn over time from each tube and quenched by the addition of 400 µL of 1:1 0.1 M MCAA/0.2 M $HClO_4$. Samples were frozen and stored at -78°C until the time of analysis.

Figure 16 graphically depicts the rapid disappearance of both WR 1065 and WR 1729 from fortified plasma stored at 4°. After 10 minutes, approximately 80% of the initial amount remains. In contrast both compounds appear to be stable at 4° in the 1:1, 0.1 M MCAA/0.2 M $HClO_4$ during the duration of the experiment (120 minutes). As expected the behavior of the internal standard parallels that of WR 1065 as is quantitatively demonstrated by the invariance of the ratio, (peak height WR 1065:peak height WR 1729) during each experiment (Table 10). This indicates both compounds are degraded at the same rate and probably are undergoing the same reactions.

b. Stability Study in Treated Plasma Stored at -78°C

(1) Stability in 1:1 0.1 M MCAA/0.2 M $HCLO_4$ at -78°C

A solution (400 µL) of WR 1065 and WR 1729, 5 and 10 µg respectively, in 0.1 M MCAA/0.2 M $HClO_4$ was placed in a plastic tube.

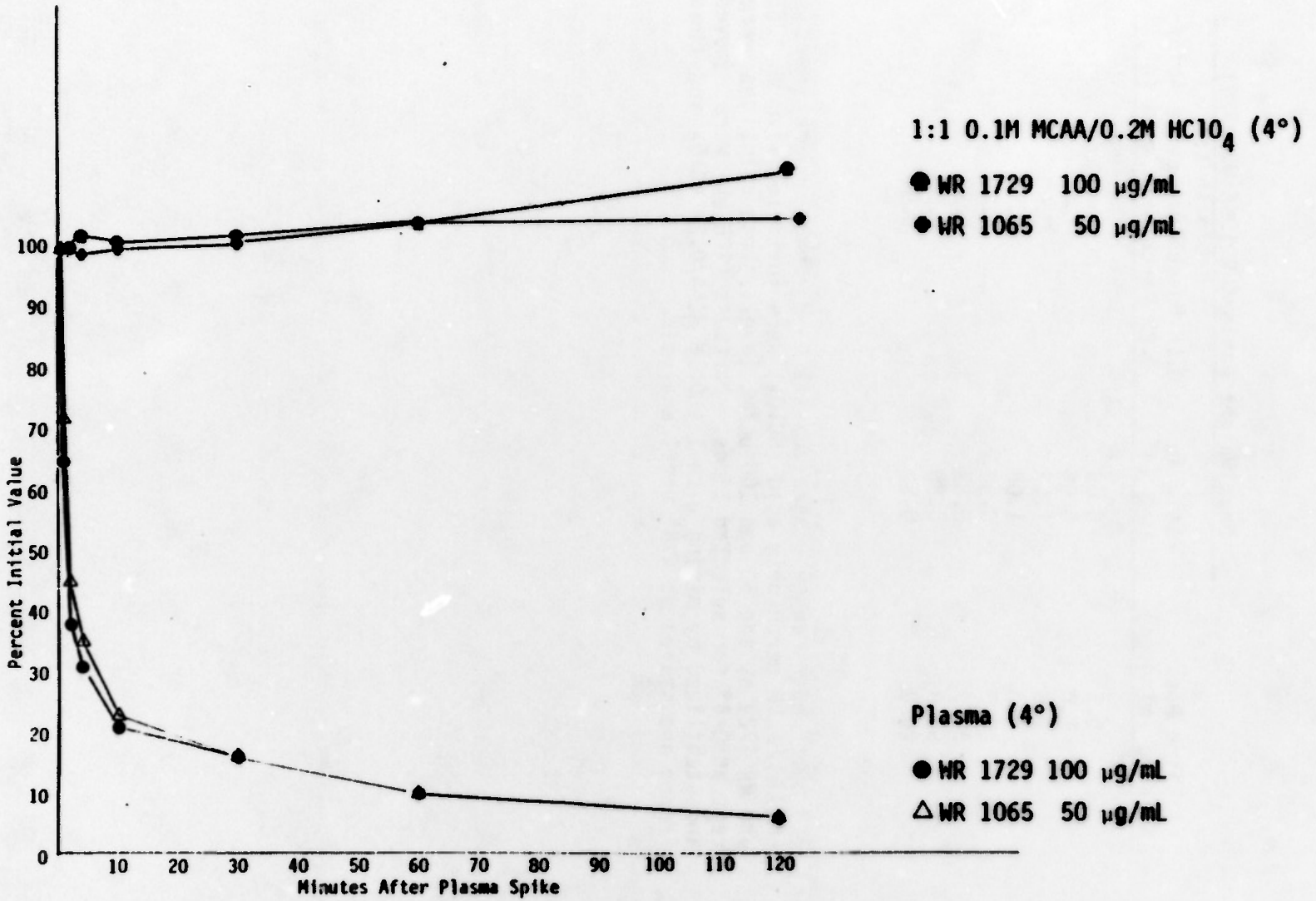


Figure 16. Stability of WR 1065 and WR 1729 in Plasma and 1:1 0.1M Monochloroacetic Acid (MCAA)/0.2M Perchloric Acid ($HClO_4$).

TABLE 10. CHANGE IN THE PEAK HEIGHT RATIO (WR 1065:WR 1729) DURING STORAGE OF FORTIFIED SAMPLES AT 4°C

Time Post Spiking (minutes)	Peak Height Ratio (WR 1065:WR 1729)	
	Ratio in Plasma (4°)	Ratio in 1:1 0.1M Monochloroacetic Acid/ 0.2M Perchloric Acid (4°)
0.5	0.88	-
1.0	0.96	-
2.0	1.03	0.90
4.0	1.00	0.86
10.0	0.94	0.89
30.0	0.89	0.88
60.0	0.88	0.90
120.0	0.80	0.84

3.0 mL of both canine plasma and 1:1 0.1M monochloroacetic acid (MCAA)/0.2M perchloric acid ($HClO_4$) were fortified with WR 1065 and WR 1729 at the 50 and 100 µg/mL level. Both solutions were kept at 0-4° for selected times. Portions (100 µL) were removed and stabilized by mixing with 1:1 0.1M MCAA/0.2M $HClO_4$ and then frozen and stored at -78° until analysis.

Canine plasma (100 µL) was added, the solution mixed, rapidly frozen in a dry-ice/isopropanol bath and stored at -78°C until the time of analysis. Periodically, samples were removed from the freezer, thawed at room temperature and analyzed.

(2) Stability Study in 1 M MCAA

Plasma (3.0 mL) was fortified with WR 1065 and WR 1729, 33 and 67 µg/mL, respectively. Fortified plasma (100 µL) was added to 100 µL of 1 M MCAA, quickly frozen using a dry-ice/isopropanol bath and stored at -78°C until the time of analysis. Single samples were removed from the freezer, thawed at room temperature and brought to 500 µL total volume by the addition of 300 µL of 0.2 M $HClO_4$. These samples were centrifuged for 10 minutes at 2500 RPM in a refrigerated (4°) centrifuge and analyzed.

(3) Summary of Stability Results at -78°C

Storage at -78°C in an acidic medium greatly reduces the rate of disappearance of both WR 1065 and WR 1729 from plasma as is shown in Figure 17. Stability is best in the mixed 1:1 0.1M MCAA/0.2 M $HClO_4$ indicating little disappearance after seven days storage. In monochloroacetic acid there is a decrease to approximately 60% of the initial concentration in 2 days with little or no change through 5-1/2 days. The peak height ratios presented in Table 11 again remain constant over the time course of the experiment supporting the choice of internal standard.

c. Synthesis of Internal Standard WR 1729·2 HCl

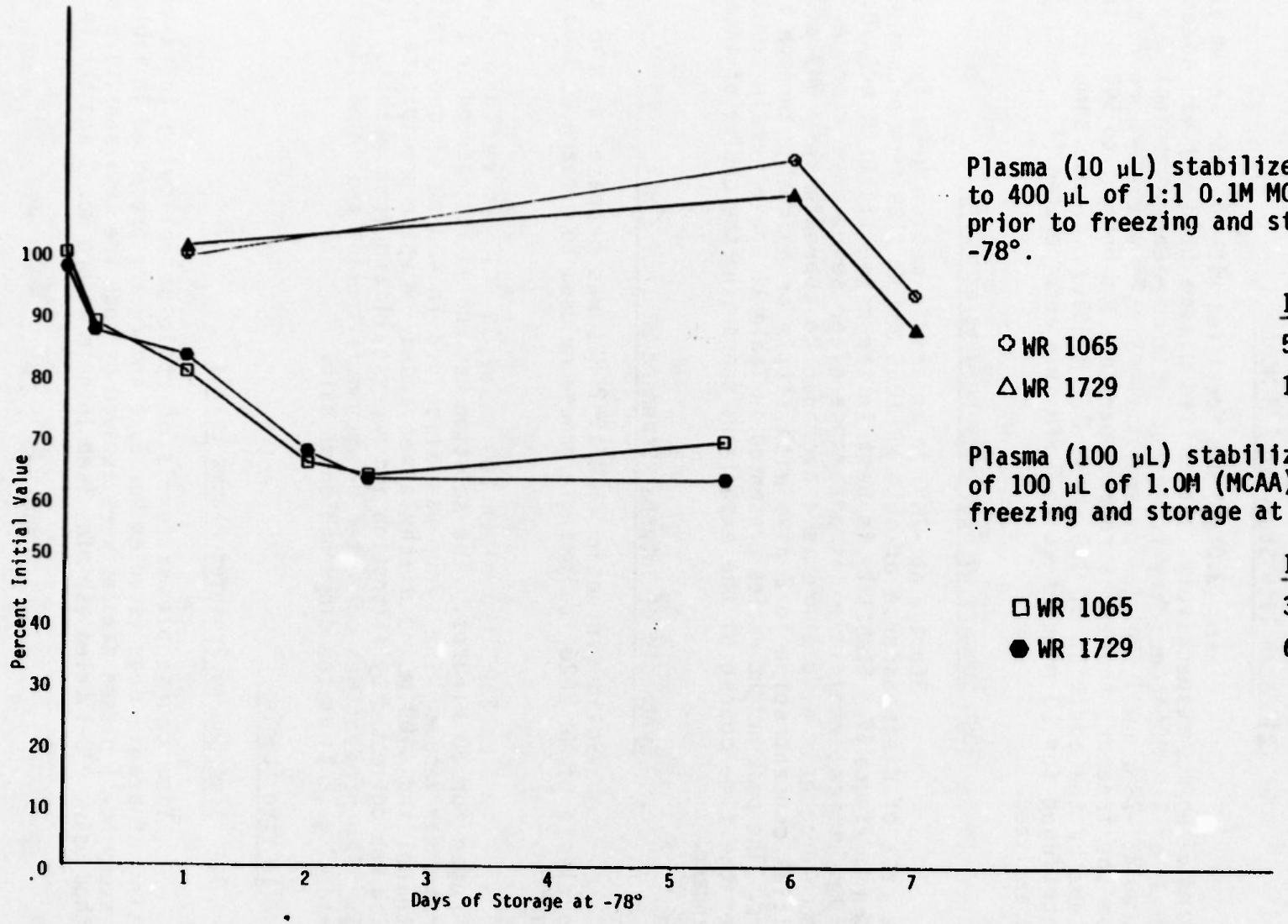
Solid synthetic WR 2719·2 HCl was prepared by the acid hydrolysis of WR 2823 following a procedure used to prepare WR 1065 from ethiofos.[2]

WR 2823 (10 g) in 100 mL of 3N HCl was refluxed under nitrogen for 30 minutes. The solution was cooled and reduced to a clear syrup under vacuum. The syrup was dissolved in 400 mL of deoxygenated methanol and ~400 mL of diethylether added. A white precipitate formed which was collected by filtration and recrystallized from methanol/ether 1/1. The product was collected by vacuum filtration and dried in vaccum. Yield ~2 g (21%) as the dihydrochloride salt.

B. In Vivo Studies

1. Time Course Ethiofos Plasma Levels

Time course plasma levels of ethiofos following intravenous dosing of beagle dogs (studies No. 5, 6 and 7) are presented in Table 12. In study No. 5 plasma samples were assayed by both the less sensitive older method using ^{14}C-labeled ethiofos (see Interim Report No. 2 and the initial



Plasma (10 µL) stabilized by addition to 400 µL of 1:1 0.1M MCAA/0.2M $HClO_4$ prior to freezing and storage at -78°.

	Initial Conc.
○ WR 1065	50 µg/mL
△ WR 1729	100 µg/mL

Plasma (100 µL) stabilized by addition of 100 µL of 1.0M (MCAA) prior to freezing and storage at -78.

	Initial Conc.
□ WR 1065	33 µg/mL
● WR 1729	67 µg/mL

Figure 17. Stability of Plasma Solutions of WR 1065 and WR 1729 at -78° After Treatment with 1M MCAA or Mixed 0.1M MCAA/0.2M $HCLO_4$ 1:1.

TABLE 11. CHANGE IN PEAK HEIGHT RATIO (WR 1065/WR 1729) DURING STORAGE OF FORTIFIED PLASMA SAMPLES AT -78°C UNDER DIFFERENT STABILIZATION CONDITIONS.

Days of Storage at -78°	WR 1065/WR 1729 Peak Height Ratio	
	Plasma Stabilized in 1:1 0.1 M MCAA/0.2M $HClO_4$[1]	Plasma Stabilized in 1 M MCAA[2]
0	-	6.1
0.25	-	6.1
1	0.63	5.9
2	-	5.9
3	-	6.1
5.4	-	6.6
6	0.66	-
7	0.68	-

1. 50 µg/mL WR 1065; 100 µg/mL WR 1729
2. 33 µg/mL WR 1065; 67 µg/mL WR 1729

TABLE 12. ETHIOFOS ANIMAL DOSING STUDIES

Dosing Study	No. 5	No. 6	No. 7
Date	2/16/83	10/4/83	10/26/83
Beagle	No. 1	No. 1	No. 2
Plasma Samples Storage	-20°C	-78°C	-78°C
Infusion Time (min)	2	2	10

Time of Blood Sampling (Post Infusion) (min)	Ethiofos (ug/mL) (a)	(b)	Ethiofos (ug/mL) (c)	Ethiofos (ug/mL) (c)
1			1051	
2	671	470	769	403
3	485	375	737	
4				297
6	335	305	609	293
9	253	230	448	224
12	195	225	323	374
15	149		358	290
22	78	86.0	199	146
30	39	36.5	130	73.6
40	9.9	220	58	36.3
50	8.8		59	17.3
60	5.9	14.0	31	7.21
70				5.10
72	3.9	3.80	13	
75		2.95	6.90	
80				2.27
84	3.9	0.90	4.55	
90				3.12
96	2.4	1.20	2.44	
100				3.62
108	3.5	1.25	3.03	
110				4.34
120	2.8	1.20	3.06	4.96
180	4.6	1.55	5.63	7.53
240	5.2	3.05	6.43	9.39
300			6.51	12.4
301	6.5	3.38		
360	5.6	3.00	6.69	12.4
480				11.2
720				91.4*
960				7.66
1380	1.7/3.5	1.85		
1440			2.81	4.05
1740	0.04	6.39		
1800			1.84	2.53
2820	-0.03	2.32		
2880			1.12	1.57
3240				1.40
4320			0.71	
7140	-0.02	0		
7200				0.43
8620				0.35
8640			0.20	

* Plasma sample hemolyzed causing interference.

(a) ^{14}C-Labeled WR 2721 as internal standard (App A and B).

(b) WR 80855 as internal standard. (The initial method developed was very similar to the latest improved method (c) except that the mobile phase was 22% acetonitrile 78% water modified with 0.01 M TBAP. This mobile phase did not allow good separation of ethiofos from endogenous materials).

(c) WR 80855 as internal standard with improved procedure (App C and D).

HPLC method developed using WR 80855 as internal standard). For dosing studies No. 6 and 7, the newly developed improved HPLC method was used (Interim Report No. 3). The time course levels of ethiofos for studies No. 6 and 7 are plotted in Figure 18. In study No. 6, a two-minute infusion time was used, while in study No. 7 the infusion was over a ten-minute period. Nevertheless the curves are very similar. The data for study No. 7 were the average values from duplicate analyses at each time period. In general very close agreement was observed between replicates which indicates that single analyses may be used with little or no loss of accuracy.

Protocols and logs for each dosing study appear in Appendix F.

2. Preliminary Pharmacokinetic Data

The data for dog dosing studies No. 6 (10/4/83) and No. 7 (10/26/83) were fit to a compartmental model with zero-order input into compartment A (the body), first-order transfer to a "delay" compartment (compartment B) and first order elimination from compartment A (see Scheme 1).

The following differential equations describe the model.

$$\frac{dA}{dt} = k_{21}\,B + R - (k_{12} + k_{10})A$$

$$\frac{dB}{dt} = k_{12}\,A - k_{21}\,B$$

where

A and B are the amounts in compartments A and B, R is the infusion rate.

k_{10} = Cle/Vc where Cle is the elimination clearance and Vc is the apparent volume of compartment A.

k_{12} and k_{21} are transfer constants from A to B and from B to A respectively. K_{21} is set equal to zero at all times before the delay is over.

This model is similar in many respects to that proposed by Steimer et al.[3]

The digital computer program NONLIN[4] was used in combination with a problem-specific subroutine. The subroutine included a fourth order Runge-Kutta numerical integration algorithm to solve the differential equations at specific times. For dog dosing study No. 6, "delays" of 90

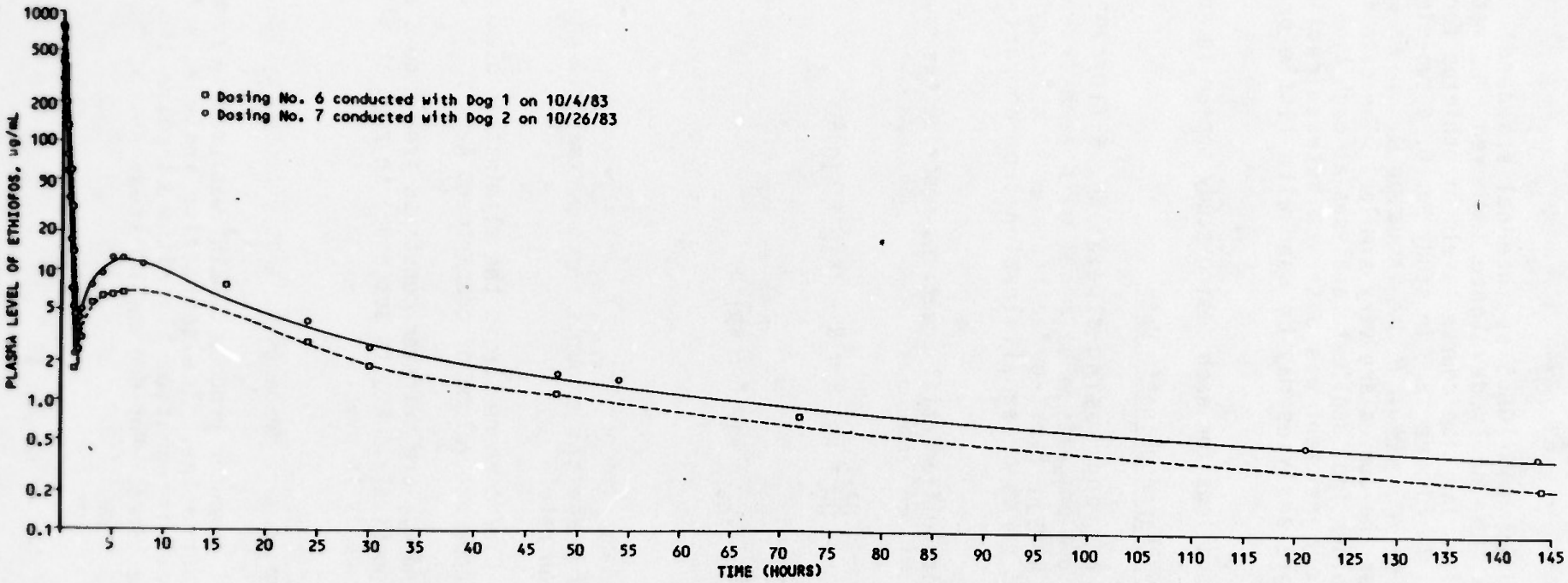
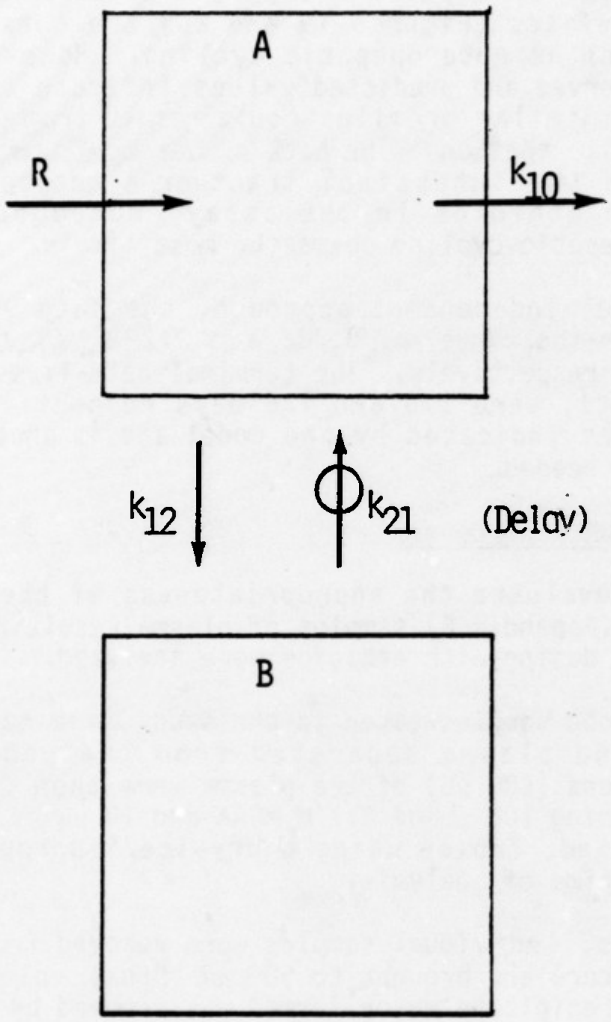


Figure 18. Beagle Plasma Level of Ethiofos Following Intravenous Dosing.



Scheme 1: Two compartment model with delay of drug return from compartment B to compartment A.

and 100 minutes were evaluated and minimal differences in the goodness of fit were noted. For study No. 7 "delays" of 100 and 105 minutes were used with similar results.

The individual parameters are given in Table 13. The overall concentration-time profiles (Figures 19 and 20) are consistent with a recycling process such as enterohepatic cycling. However, systematic deviations between observed and predicted values indicate the need for a more complex model. Similar profiles could result from production of a long half-life metabolite that could be back converted to ethiofos in the body rather than in the intestinal tract or a metabolite that co-chromatographs with ethiofos in the assay procedure. Of these possibilities, enterohepatic cycling seems the most likely.

Using a model independent approach, the data yield clearance values (dose/area-under-the-curve) of 0.332 and 0.298 L/hr/kg for dosing study No. 6 and No. 7 respectively. The terminal half-lives, based on the last 3 or 4 data points, were 1.5 and 1.6 days respectively. This is longer than the values indicated by the model and is another indication that a better model is needed.

3. Time Course WR 1065 Levels

In order to evaluate the appropriateness of the assay method developed for WR 1065 (Appendix E) samples of plasma resulting from the dog study No. 8 (12/14/84) dosing with ethiofos were analyzed.

The whole blood samples taken in the study were rapidly chilled in ice water and the plasma separated from the packed cells by centrifugation. Portions (100 µL) of the plasma were then transferred to plastic tubes containing 100 µL of 0.1 M MCAA and 10 µg of WR 1729. This mixture was rapidly mixed, frozen using a dry-ice/isopropanol bath and stored at -78°C until time of analysis.

For analysis, individual samples were removed from the freezer, thawed at room temperature and brought to 500 µL final volume with 0.2 M $HClO_4$. Any protein precipitate which formed was removed by centrifugation prior to sample analysis. Portions (20 µL) were subjected to chromatographic analysis by the assay method as described in detail in Appendix E.

Table 14 and Figure 21 presents the WR 1065 concentrations found in the stored plasma samples upon analysis. The maximum concentration, 38.2 µg/mL occurs at 15 minutes post infusion with a gradual decrease to approximately 1 µg/mL by 189 minutes post infusion. Some WR 1065 is still present 720 minutes (~12 hours) post infusion (~0.14 µg/mL) but little or none is detectable in samples from later time points. These data indicate WR 1065 lingers in the plasma some time after the initial ethiofos dose, but quantitative interpretation should be viewed cautiously in light of reported short term stability at 4°C which suggests the values here reported may represent as little as 10-20% of the actual WR 1065 present in the canine plasma at the moment the blood is collected.

TABLE 13. PARAMETER VALUES

Parameter	Dog Study No. 6		Dog Study No. 7	
	90 min delay	100 min delay	100 min delay	105 min delay
V_c, L/kg	0.152	0.153	0.249	0.250
K_{12}, hr^{-1}	1.70	1.69	2.21	2.20
K_{21}, hr^{-1}	0.0482	0.0563	0.0840	0.0918
Cle, L/hr/kg	0.357	0.356	0.341	0.342

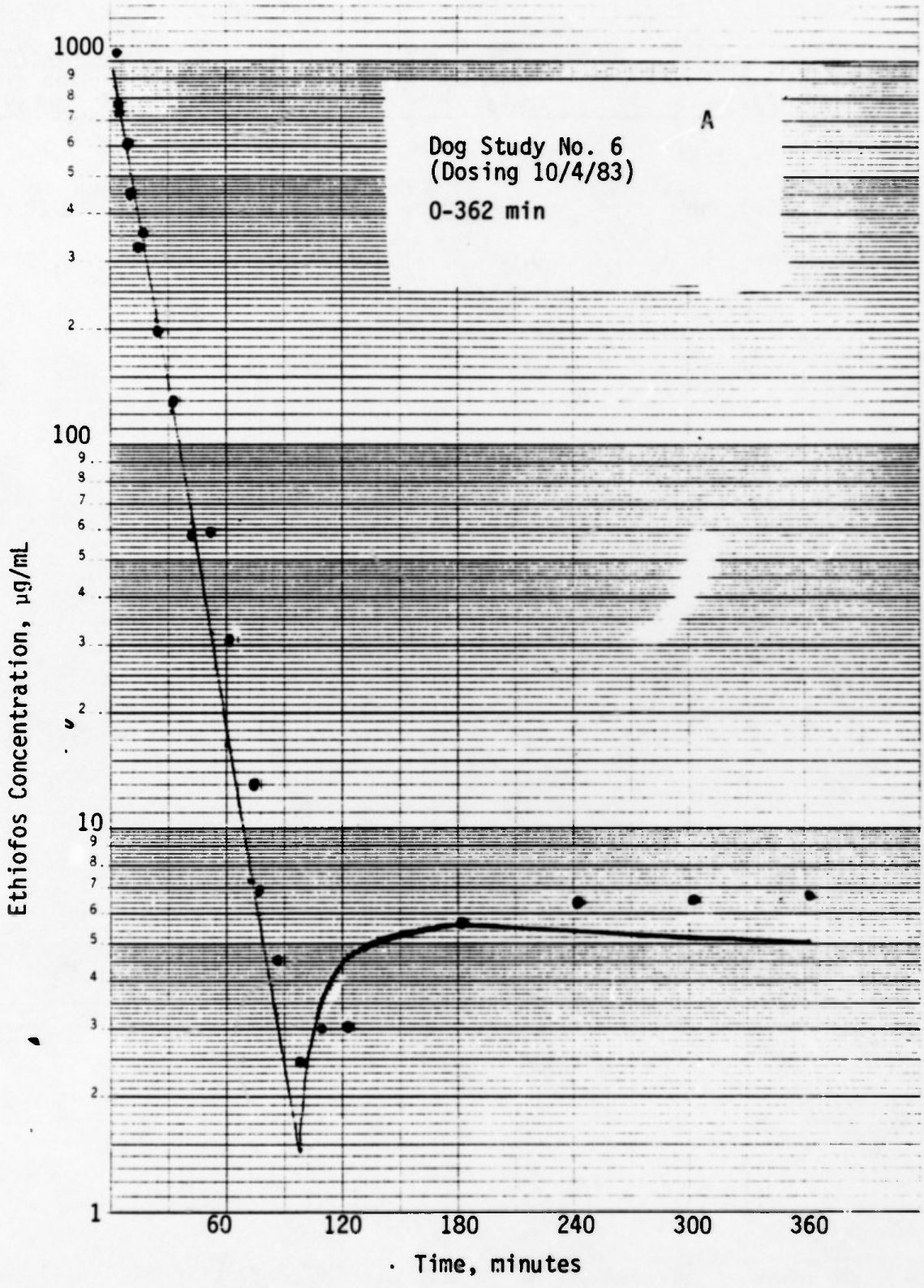


Figure 19a. Concentration Time Profile for Dog Study No. 6. The Line Represents the Profile Predicted by the Model (see text).

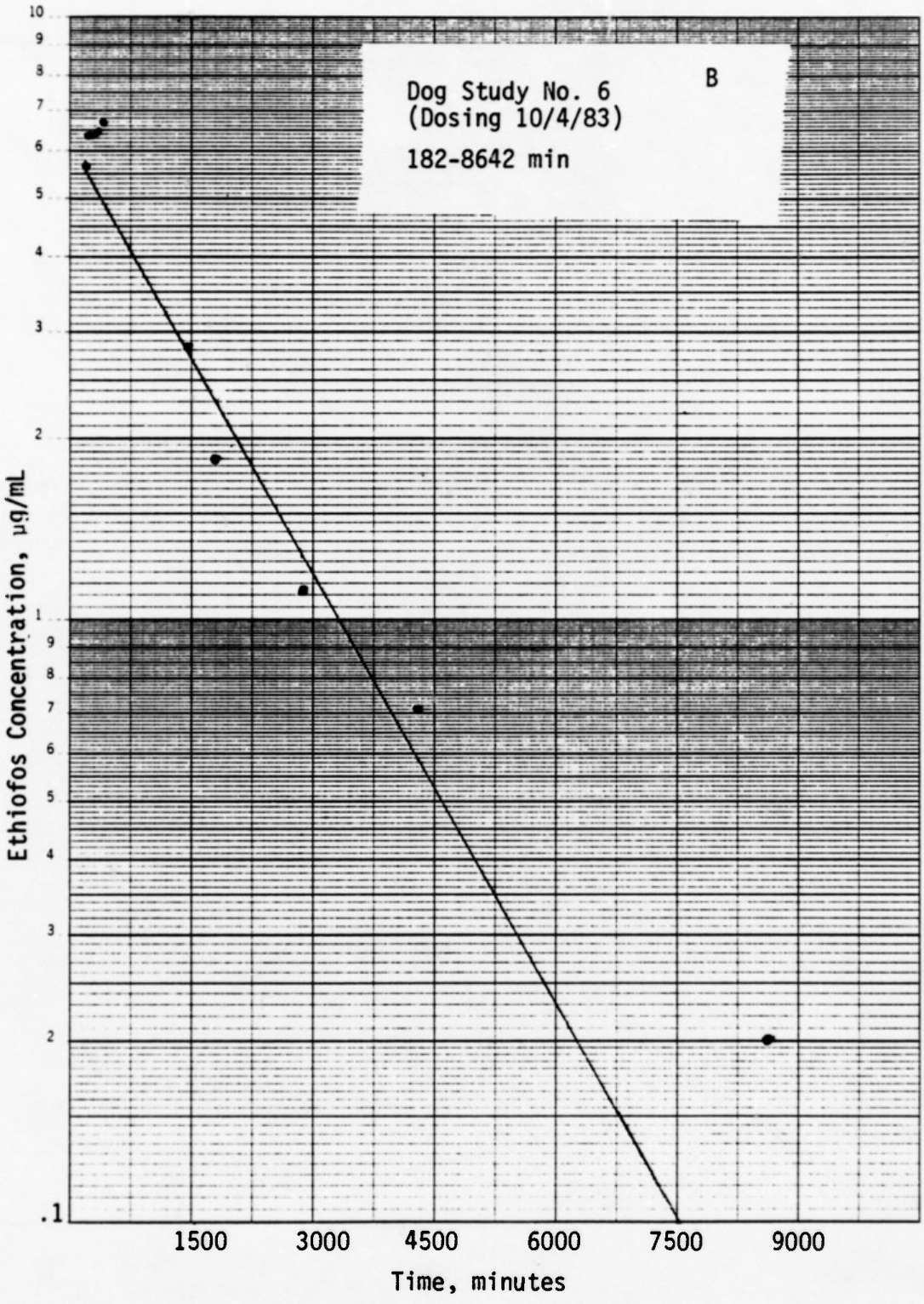


Figure 19b. Concentration Time Profile for Dog Dosing No. 6. The Line Represents the Profile Predicted by the Model (see text).

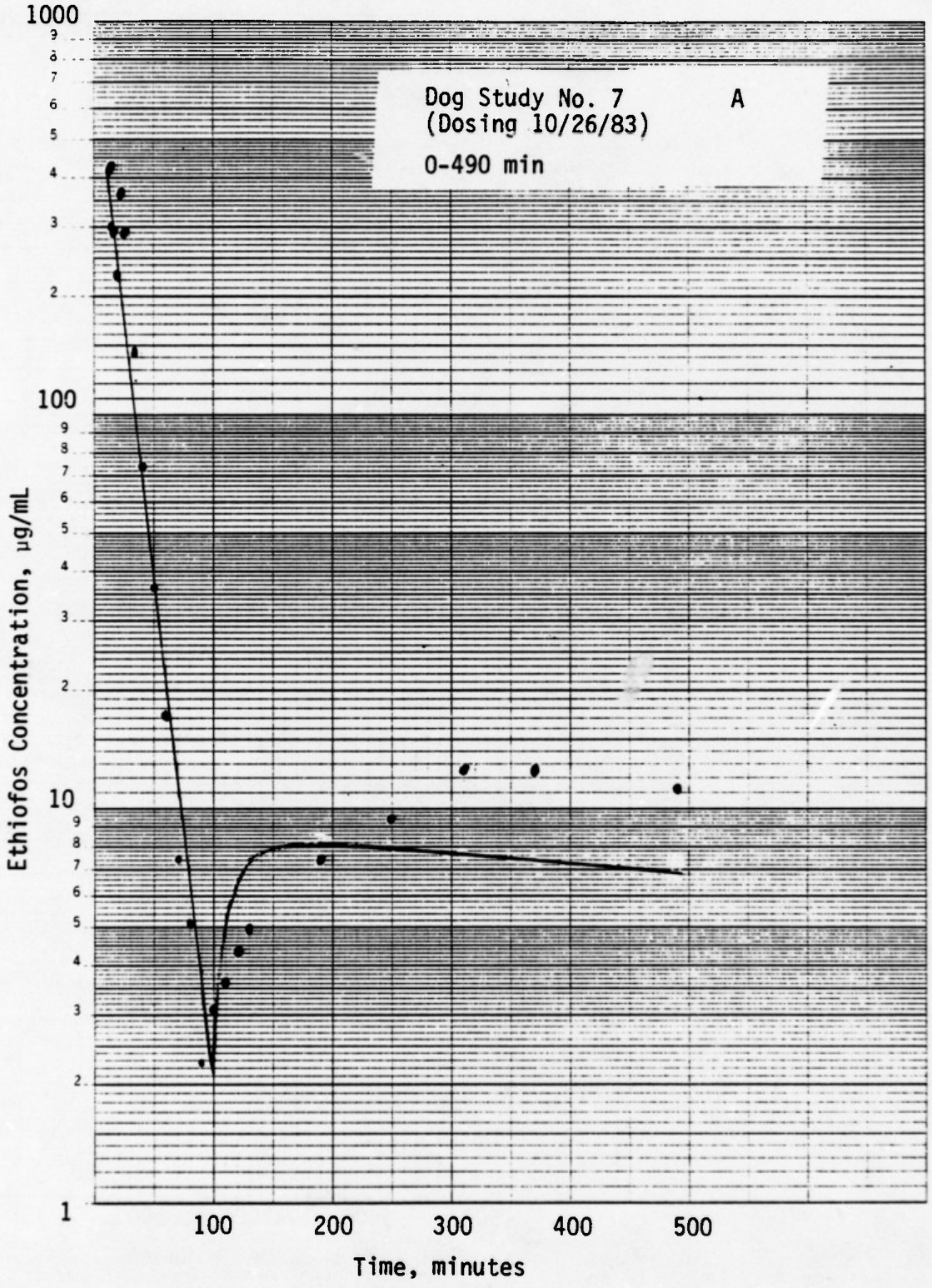


Figure 20a. Concentration Time Profile for Dog Study No. 7. The Line Represents the Profile Predicted by the Model (see text).

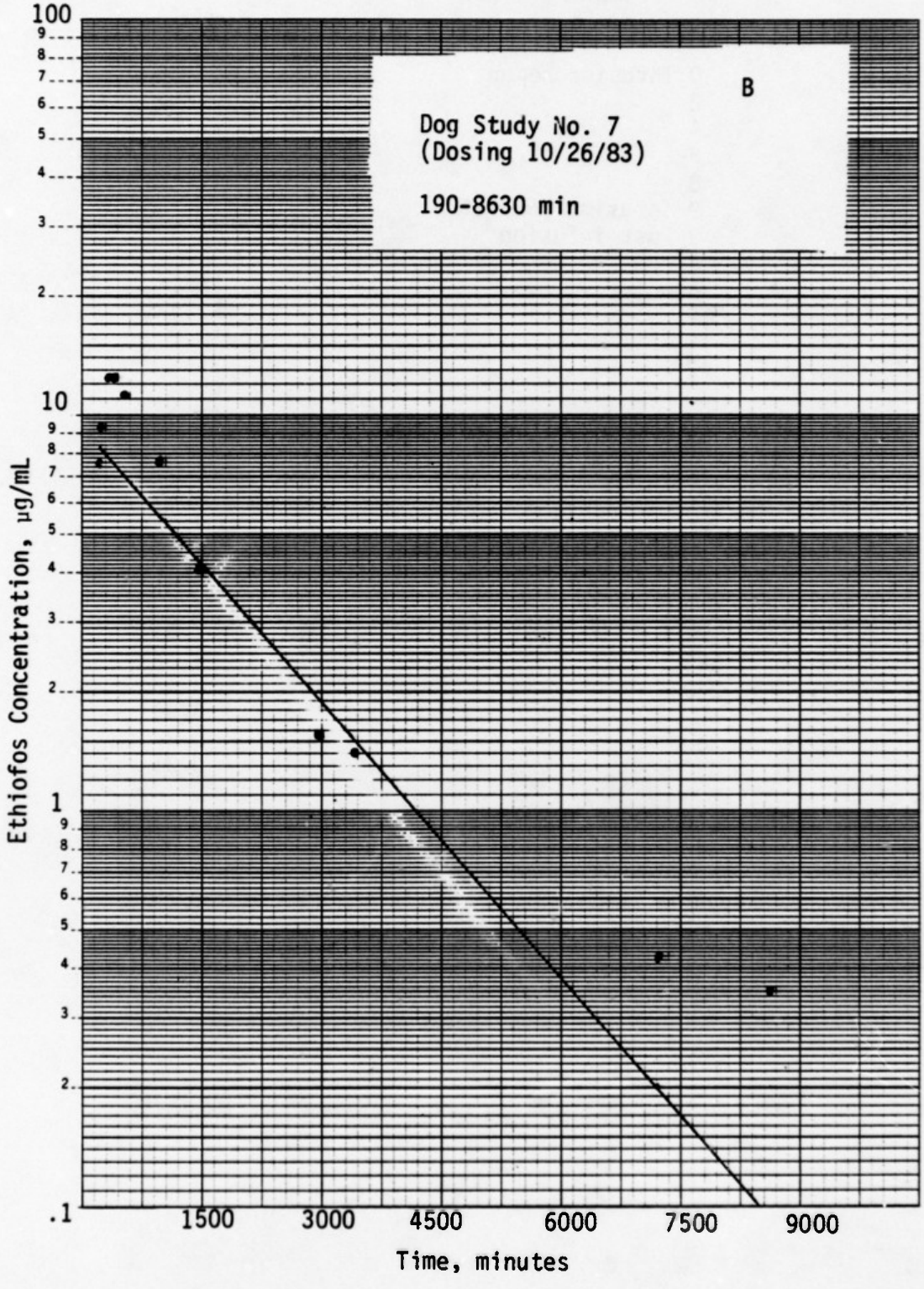


Figure 20b. Concentration Time Profile for Dog Study No. 7. The Line Represents the Profile Predicted by the Model (see text).

TABLE 14. ETHIOFOS DOG DOSING STUDY NO. 8 (12/14/83)

Sampling Time (min)		Plasma Levels WR 1065* (µg/mL)
0	Infusion begun	
2		4.88
4		15.5
6		19.0
8		31.5
9	Infusion completed	-
2	Post infusion	32.0
4		30.8
6		34.6
12		32.8
15		38.2
22		34.9
30		29.8
40		27.8
50		23.3
60		12.3
70		9.74
80		9.01
90		6.05
100		5.01
110		3.50
121		2.64
189		1.23
249		0.42
300		0.38
360		0.30
480		0.21
720		0.14
960		0
1440		0
1800		0
2880		0
3240		0
7200		0
8640		0

*Determined by method as reported in Appendix E.

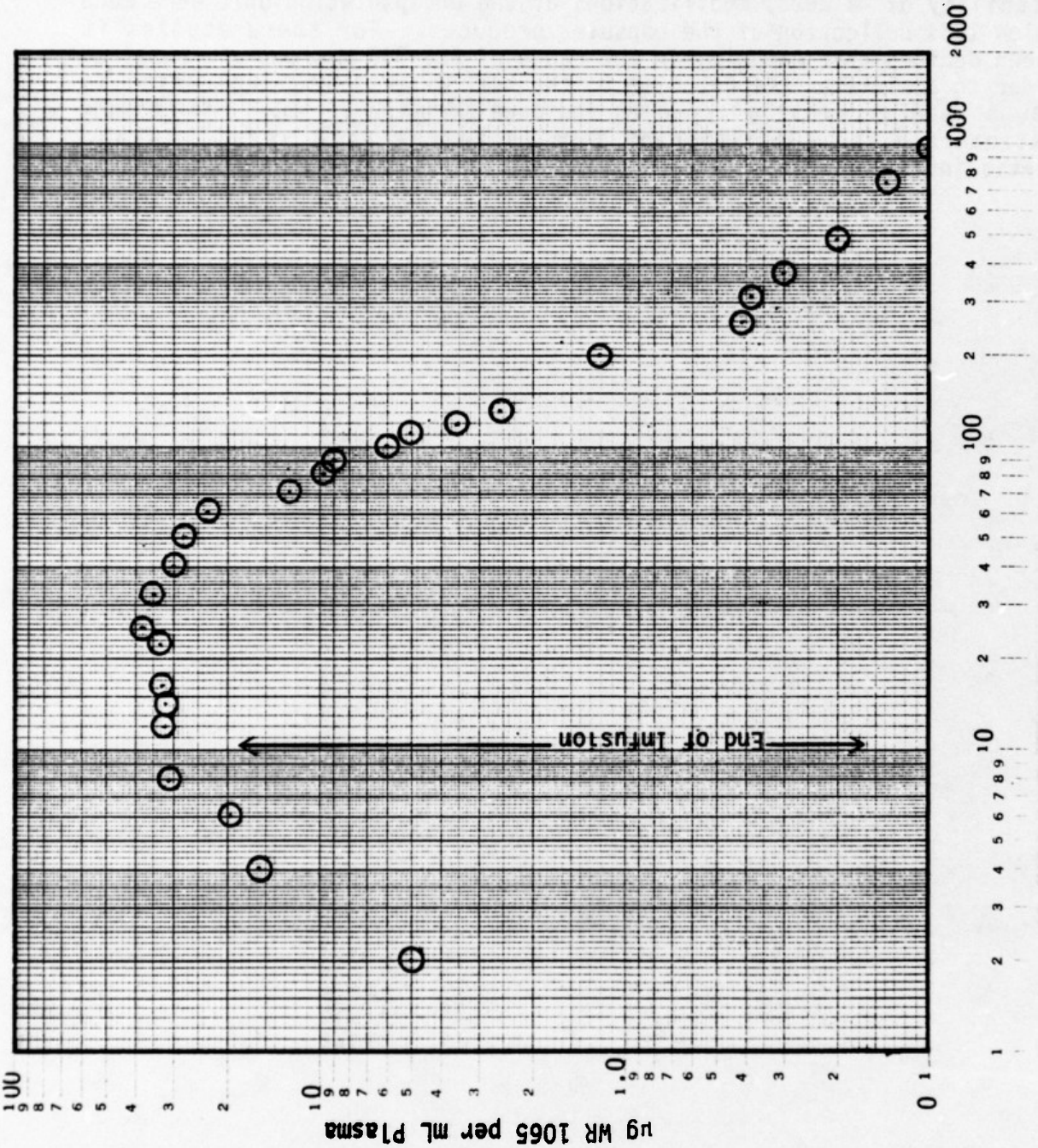

μg WR 1065 per mL Plasma
End of Infusion
Time in Minutes from Start of Infusion.

Figure 21.

C. Microencapsulation

Microencapsulation runs were again initiated near the very end of this report period to develop operating conditions for the encapsulation of WR 2823 for special animal dosing studies at WRAIR. Due to the limited availability of WR 2823, modifications of the encapsulation unit were made to allow 100% collection of the capsules produced. For these studies it has been decided that capsules in the range of 425-600 microns are required in order to administer the capsules to the test animals by gavage using an 18-gauge needle. Initial studies indicate that the capsules can be made satisfactorily and evaluation of samples in the acid stability and synthetic intestinal fluid release tests are in progress.

V. DISCUSSION

A. Analytical Methods Development

1. Ethiofos

a. ^{14}C-Labeled Ethiofos as Internal Standard

The HPLC method initially developed for ethiofos assay had good precision and accuracy over all but the lowest portion of the concentration range. In this region the amount of the drug originally present becomes comparable to the uncertainty associated with the measurement of the radiolabeled internal standard. The amount of the standard which was added could not be further reduced unless a standard with higher activity was made available. This approach was judged to be impractical and a decision was made to search for a different internal standard and an appropriate chromatographic system.

b. WR 80855 as Internal Standard

A homolog of ethiofos, S-3-(4-aminobutylamino)propyl phosphorothioate (WR 80855) was chosen as the internal standard for a revised HPLC assay because of its chromatographic behavior and similar chemical properties to ethiofos. Although the fluorescamine derivatives of the two esters differ by only two methylene units, the resolving power of the RCM-100 5μ C-18 reverse phase system is capable of baseline separation of the chromatographic peaks. At least two other brands of C-18 steel filled colums have been found to do the same. These columns are 4.6 mm in diameter (versus 8 mm) and restrict the flow rate to <1.2 mL/min. A higher rate is desired to flush the column in a minimum length of time.

About the time that characterization of the WR 80855 internal standard method was begun a new fluorescence detector was found which increased sensitivity roughly twentyfold. This immediately lowered the minimum detectable limit (MDL) to less than 0.05 μg ethiofos per mL of plasma. (MDL is defined as the lowest amount which can be positively identified and quantitiated within acceptable limits of precision and accuracy.) The new sensitivity revealed a new, small peak in the chromatogram which co-eluted with ethiofos. This interference varied from an ethiofos equivalent of 0.1-0.7 μg/mL on four beagle plasma samples. A simple change in the mobile phase from 22/78 acetonitrile/water to 16:7:77 acetonitrile/ethanol/water (both 0.01 M in TBAP) caused the interference to be separated from the peaks of interest at the expense of a ten minute increase in elution times. The ternary mobile phase was used for the method characterization studies. The assay has been found to be suitable in each of the dosing studies described in this report with excellent agreement obtained among replicate samples, however, a potential problem has been identified; a peak due to an endogenous component in the plasma which elutes immediately before ethiofos was found in several instances to increase in size to the extent that resolution between the two was lost.

This interference is a problem only with the lower levels of ethiofos but it is at these levels that most of the in vivo samples fall. The interference was not a problem with the vast majority of the samples; however, modifications to the chromatographic system are being investigated which are intended to increase the separation between the two peaks.

2. WR 1065

Further long term plasma stability investigations at -78° are in progress to more accurately characterize the behavior of both WR 1065 and WR 1729 during storage conditions.

The short term plasma stability study makes it apparent that samples must be processed quickly up to the point of internal standard addition. This may mean adding the internal standard to whole blood before plasma separation, or if satisfactory, rapidly (within 1-2 minutes of sampling) separating the plasma and adding internal standard.

The constancy of the peak height ratio of WR 1065:WR 1729 during storage supports the choice of this compound as an internal standard for this study.

B. In Vivo Studies

The in vivo data analysis and its interpretation should be considered as preliminary since other models need to be explored. Use of a two- instead of a one-compartment body model with an input delay[5] will probably describe the data better. These analyses will be done in the near future. A more complex model may also be required when data following oral dosing becomes available. In addition, studies in bile cannulated dogs would help define the model more completely.

C. Microencapsulation

Microencapsulation experiments with ethiofos were not undertaken during this report period in any significant amount due to the inability to conduct oral dosing studies because of the lack of blood assay methods. With the recent development of the assay methods, oral dosing should be initiated in the very near future.

VI. CONCLUSIONS

1. Ethiofos in plasma can be readily quantitated over the range of 0.05 to >1000 µg/mL using an HPLC procedure with WR 80855 as an internal standard.

2. The developed assay can be applied successfully in ethiofos dosing experiments using beagle dogs. Preliminary evaluation of the results indicates the data is compatible with a recycling process with a clearance of about 0.3 L/hr/kg and an apparent half-life of about 1.5 days.

3. WR 1065 in plasma can be quantitated over the range of 1 to >500 µg/mL using an HPLC procedure with WR 1729 as an internal standard.

4. WR 1065 disappears readily in plasma even at -78°C, however, it appears that plasma samples containing the compound can be stabilized sufficiently to allow for assay.

5. The developed WR 1065 assay procedure can be used for the assay of plasma samples from a ethiofos dosing study, however, the initial results must be considered tentative until the stability factors are resolved.

VII. RECOMMENDATIONS

1. Initiate ethiofos oral dosing studies to evaluate, through bioavailability and pharmacokinetic studies, the most promising encapsulated dosage forms.

2. Improve the WR 1065 HPLC assay method to cover the approximate range of ~0.05 to >500 µg/mL using WR 1729 as an internal standard.

3. Develop an HPLC assay method for WR 33278, the disulfide metabolite of ethiofos.

4. Evaluate developed assay methods for WR 1065 and WR 33278 by IV dosing using the beagle dog as animal model.

5. Further optimize dosage formulation(s) of ethiofos.

6. Develop sustained-release dosage forms of ethiofos.

7. Continue in vitro evaluation of promising microcapsules by determining stabilities in acid solution and release rates in synthetic intestinal fluid.

8. Continue determination of aging effects at 25°C and 37°C on the primary encapsulated samples.

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APPENDIX A

Interim Report No. 2
HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate
(WR 2721) in Plasma

AD _____

Interim Report 2
HPLC Assay For S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) In Plasma

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Nollie F. Swynnerton, Ph.D., Senior Research Scientist
Edward P. McGovern, Ph.D., Senior Research Scientist
Joe A. Nino, Senior Technician
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February 1983

Support By
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

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Southwest Research Institute
6220 Culebra Road
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This report contains a description of an analytical procedure for the determination of WR 2721 in plasma.

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AD _____

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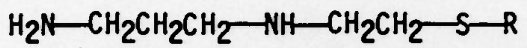
INTRODUCTION

The chemical radioprotector S-2-(3-aminopropylamino)ethyl phosphorothioate (1) has been the object of intense study by research groups of the U.S. Army and the National Cancer Institute. An Army program established in 1959 found that the drug [labeled Walter Reed (WR)-2721] protects various tissues from radiation.[1] It was also demonstrated that certain solid animal tumors are not protected, which suggested its use in radiotherapy.[2]



1, WR 2721, R = $PO(OH)_2$

2, WR 1065, R = H

It is believed that in the body the drug is transported intact into the tissue where it is enzymatically cleaved to yield the thiol 2 (WR 1065).[3] Free radicals produced by radiation are then scavenged by interaction with the sulfhydryl (SH) group of 2.[4]

A major shortcoming of WR 2721 is that its activity after oral dosing is severely limited--probably due to premature cleavage of the phosphate group under the acidic conditions encountered immediately after oral dosing.[5]

Attempted bioassay of WR 2721 has been hampered by several serious obstacles. The compound is acid-labile, has no convenient chromophore, has essentially no solubility in organic solvents and is extremely polar, capable of existing as a dipolar ion or a bis-dipolar ion. Lack of solubility precludes its extraction from biological fluids and its polarity places limits on the types of chromatographic systems which might be used to separate it from endogenous materials. To facilitate pharmacokinetic studies of the drug, an analytical procedure capable of efficiently processing large numbers of samples is also an important consideration. An HPLC method which overcomes the stated problems and is capable of handling multiple samples is presented in this paper.

MATERIALS AND METHODS

Instrumentation

A Waters Associates Model 244 Liquid Chromatograph equipped with a Model 420AC Fluorescence Detector and Data Module was employed. Excitation wavelength was 395 nm and emission wavelength was >460 nm. Samples were injected using a Rheodyne Model 7125 Injector fitted with a 20- or 50-µL loop. Separations were carried out on a Waters Associates RCM-100 Radial Compression Module fitted with a 100 mm X 8 mm cartridge filled with 5- or 10 µm spherical C-18 packing. The analytical column was protected with a Whatman, Inc. guard column filled with Waters Associates CoPell C-18 packing. The mobile phase was acetonitrile/water (22:78), 0.01M in dibutylammonium phosphate (pH ~ 3) at a flow rate of 2.0 mL/min.

Reagents

Acetonitrile was purchased from Burdick and Jackson Laboratories, Inc. Dibutylamine and fluorescamine were obtained from Aldrich Chemical Company. Concentrated (1M) solutions of dibutylammonium phosphate were prepared by titrating 12.9 g of dibutylamine to pH 2.5 with phosphoric acid and diluting to volume. Fresh solutions containing fluorescamine (5 mg/mL) were prepared weekly using reagent grade acetone which had been stored over 4A molecular sieves. WR 2721 Trihydrate, Lot AU, BJ09506, AJ-68-2 was furnished in >99% purity by the Walter Reed Army Institute of Research. Radiolabeled WR 2721, S-[2-(3-aminopropylamino)ethyl-1,2-^{14}C]phosphorothioate was obtained from Research Triangle Institute, Lot No. 3874-52; its specific activity was 86.0 µCi/mg and reported purity was >97.0%.

Standards containing WR 2721 were prepared by dissolution in 0.05 M sodium borate-potassium chloride pH 10 buffer.

Sample Preparation

Canine plasma (90 µL), 50 µL of the internal standard and 160 µL of 0.05 M sodium borate-potassium chloride pH 7.6 buffer were placed in a polyethylene vial and, while the mixture was being agitated using a vortex mixer (American Scientific Products), 200 µL of the fluorescamine reagent was added. After mixing for 60 s, the mixture was treated with an additional 200 µL of fluorescamine reagent, and agitation was continued for 20-30 s. The resulting mixture was centrifuged at 1500 rpm for three minutes and an aliquot of the supernatant was immediately injected onto the HPLC column. Each frozen sample was individually thawed, derivatized and immediately injected because both WR 2721 and its derivative decompose in plasma at room temperature.

Internal Standard

Canine plasma samples were spiked with increasing amounts of WR 2721, mixed with internal standard and derivatized and injected as described

above. The following concentrations were used to construct a standard curve: 0, 1.89, 6.33, 11.9, 56.3, 112, 279, 556, 834, and 1110 µg/mL. The curve was constructed by fitting a regression line to the peak area versus concentration data after correction for recovery. Recovery was determined by adding 50 µL of a solution containing ^{14}C-labeled WR 2721 (activity 334 dpm/µL) to each sample vial prior to derivatization. After derivatization, a 20- or 50-µL sample was removed from the reaction vial, mixed with scintillation fluid and counted using a Packard Tri-Carb Model 4530 scintillation counter. Similarly, either a 20- or 50-µL sample was analyzed using HPLC. Fractions of column effluent were collected at times bracketing the retention time of WR 2721. Each vial, after addition of scintillation fluid, was counted for 10 minutes, and the recovered radioactivity was determined by summing the disintegrations observed in the vials with activity greater than background levels. Recovery of WR 2721 was then determined for each assay using the expression: % recovery = (dpm recovered x 100)/dpm added. All calculations were corrected for contributions due to background. Counting efficiencies were determined via automatic external standardization. Recoveries obtained were between 35% and 55% (mean = 48%, N = 58).

Animal Dosing Experiments

A healthy, one-year old, AKC-registerable, male beagle dog weighing 12.7 kg was used in pilot dosing experiments to test the analytical method. The animal received intravenously via the cephalic vein a 0.9% saline solution containing 1.908 g (150 mg/kg body weight) of WR 2721. The infusion required two minutes. At periodic intervals over 6 hours, blood samples (3 mL) were drawn into EDTA Vacutainers® from a cannula placed in the jugular vein. Each sample was immediately chilled in an ice/water bath and then centrifuged. A 90-µL aliquot of the separated plasma was added to 50 µL of the internal standard, the mixture was agitated using a vortex mixer and was then quick-frozen in a dry ice/alcohol bath. Samples were stored at -20°C until time of analysis when they were thawed at room temperature and treated as described in the Sample Preparation section. The drug was shown to be stable in plasma up to about 30 days when stored at -20°C or lower, but was unstable at room temperature.

RESULTS AND DISCUSSION

Derivatization

Major obstacles in the development of the assay were the instability of WR 2721 in acidic media and its lack of a suitable chromophore. A pH-hydrolysis rate profile (Figure 1) demonstrates the lack of stability under acidic conditions.[6] The data were generated by measuring the rate of appearance of WR 1065[7] and calculating the rate of disappearance of WR 2721.

In simple aqueous systems underivatized WR 2721 has been analyzed by HPLC using separation on a Whatman PAC column[8] and UV detection at 204 nm, but this procedure was unsuitable for plasma analysis. Derivatization of the drug with fluorescamine allows detection in the picomole range and at the same time modifies its chromatographic behavior such that it is retained on reverse-phase columns.

It was found that precision was remarkably improved by a two-stage derivatization procedure (see Methods section). This may be attributed to the effect of the acetone solvent which by protein denaturization could release bound material. Pre-treatment of plasma with methanol, ethanol or acetonitrile did not provide a satisfactory increase in precision.

Derivatization with o-phthalaldehyde was briefly investigated but was not further pursued when the derivative was found to be very unstable.

Separation

Figure 2a illustrates a typical chromatogram for a plasma sample spiked with WR 2721 at the 5 µg/mL level. Note that the peak of interest is baseline-separated from those peaks due to endogenous materials, thought to be amino acids. Figure 2b illustrates a typical chromatogram of derivatized canine plasma.

The separation was performed under isocratic conditions to increase both sample throughput and precision. As a result, less polar materials (including WR 1065) are not eluted and, when allowed to remain, degrade resolution and eventually destroy the column. Flushing the column with methanol/water (70:30) at the end of each analysis day restored column resolution and increased its lifetime.

Internal Standard

The complex chromatogram of derivatized plasma did not offer much hope of finding a suitable internal standard, i.e., one which would have similar behavior toward hydrolysis during sample storage and workup, and elute in a reasonable time in an unobstructed region of the chromatogram. A number of homologs of WR 2721, several other phosphorothioates and a number of amino

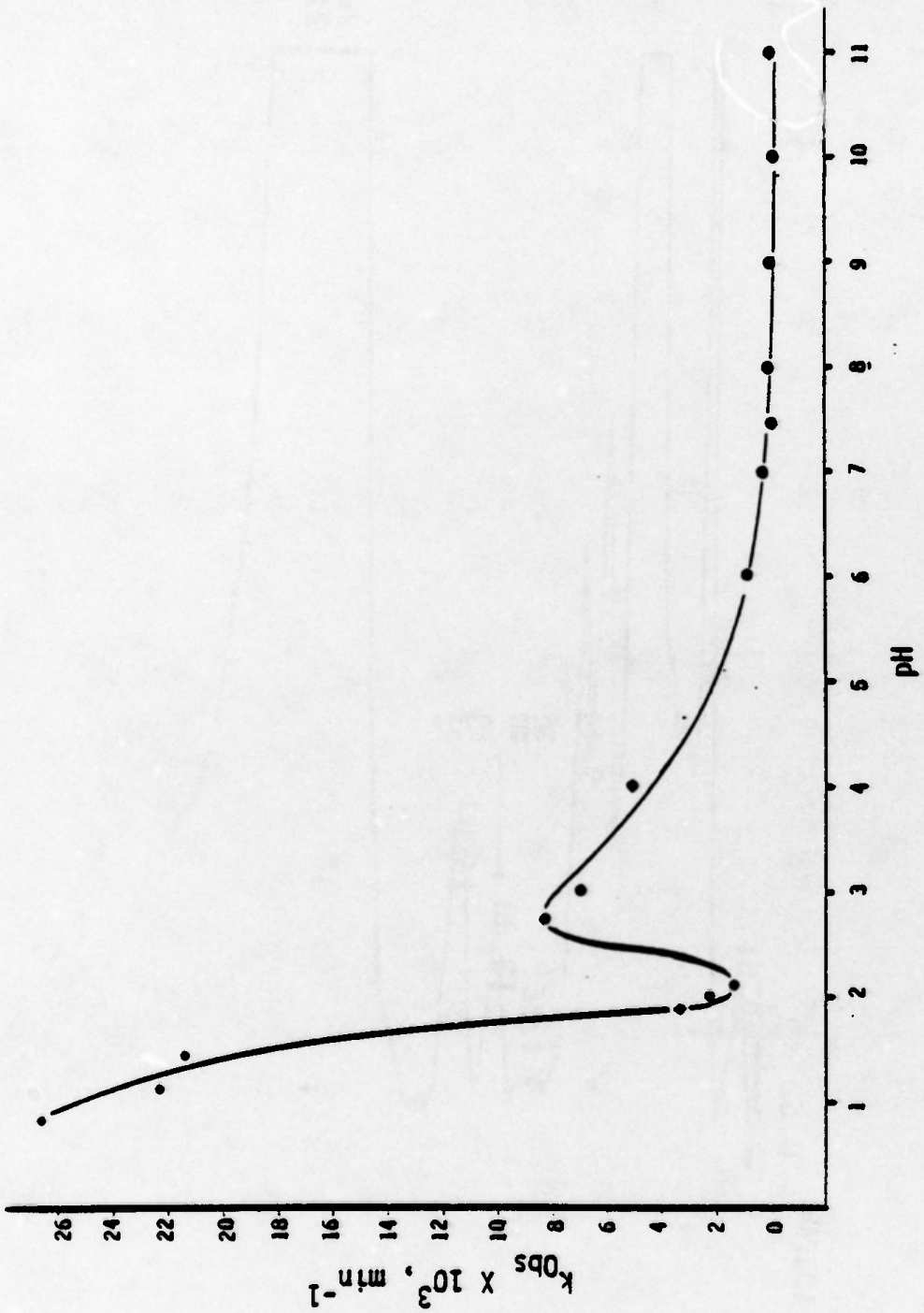


Figure 1. pH - Rate Profile for Hydrolysis of WR 2721 at 37°C.

Figure 2a. Chromatogram of WR 2721 in Beagle Plasma (5 µg/mL).

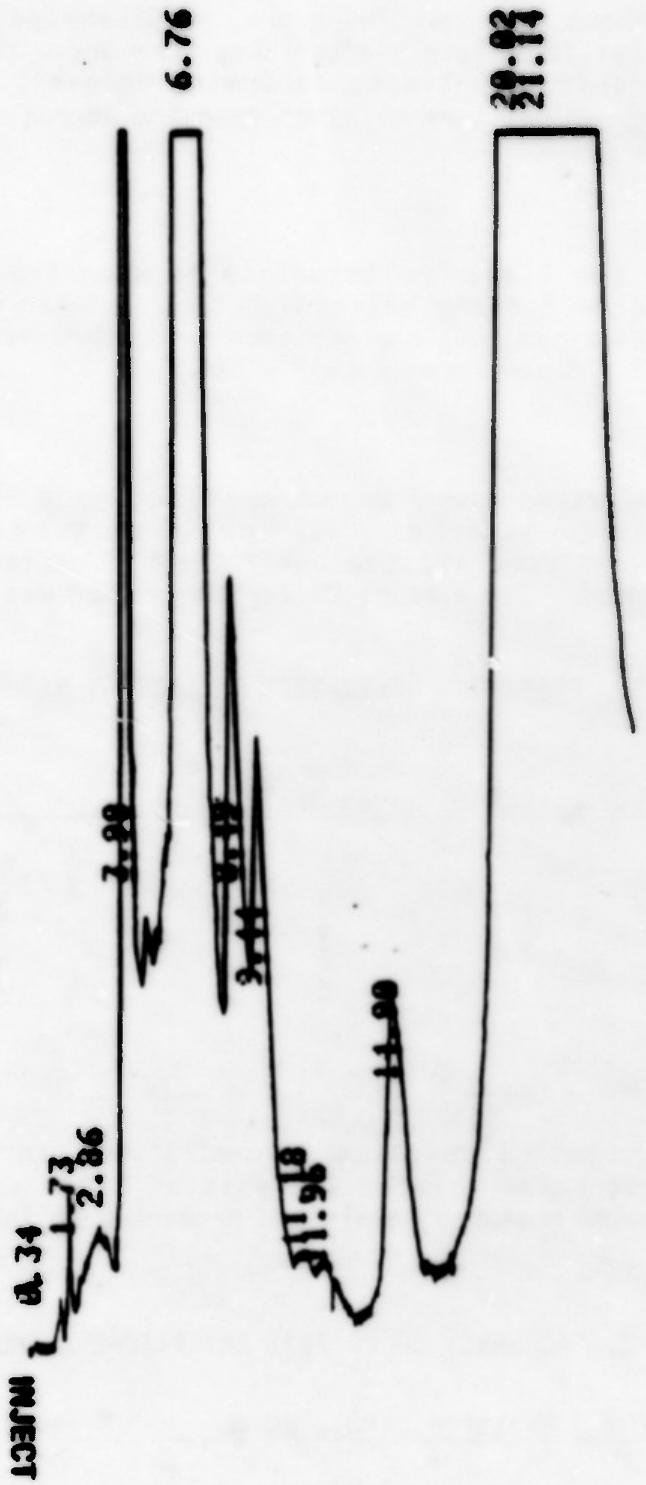


Figure 2b. Chromatogram of Beagle Plasma (blank).

acids were screened without success. Therefore, radiolabeled WR 2721 was added to plasma samples immediately after they were drawn and the column effluent was collected and counted to determine recovery. Excellent precision and accuracy values were obtained over the approximate range of 2-1100 µg/mL using this procedure.

Linearity

Figure 3 depicts the linear relationship between the drug plasma levels and the peak area for derivatized WR 2721. Linear regression of peak area versus concentration gave a coefficient of determination (R^2) of 0.9969, with a slope of 930 and intercept of -7684.

Precision and Accuracy

Precision of the method over the entire working range was determined by the analysis of replicate spiked samples. In Table 1 the concentration number of replicates analyzed and the coefficient of variation (CV) for each data set is presented. The average CV for the method was 6.6%.

TABLE 1. PRECISION OF WR 2721 ANALYTICAL METHOD

WR 2721 Concentration, µg/mL	Number of Replicates Analyzed, N	CV (%)
1110	5	9.1
112	5	5.3
11.9	5	5.6
6.3	5	5.1
3.0	5	7.8

The accuracy of the method for plasma concentrations ranging from 1.89 to 1110 µg/mL was determined by the analysis of blind spiked plasma samples. Spike levels and measured levels are presented in Table 2.

TABLE 2. ACCURACY OF WR 2721 ANALYTICAL METHOD

Spike Level, µg/mL	Measured Level, µg/mL	% Deviation
1.89	1.92	1.59
8.55	8.75	2.34
34.1	33.1	-2.96
78.6	75.9	-3.37
112	107	4.46
390	430	10.2
779	742	-4.75
1110	1050	-5.41

Average = 4.35

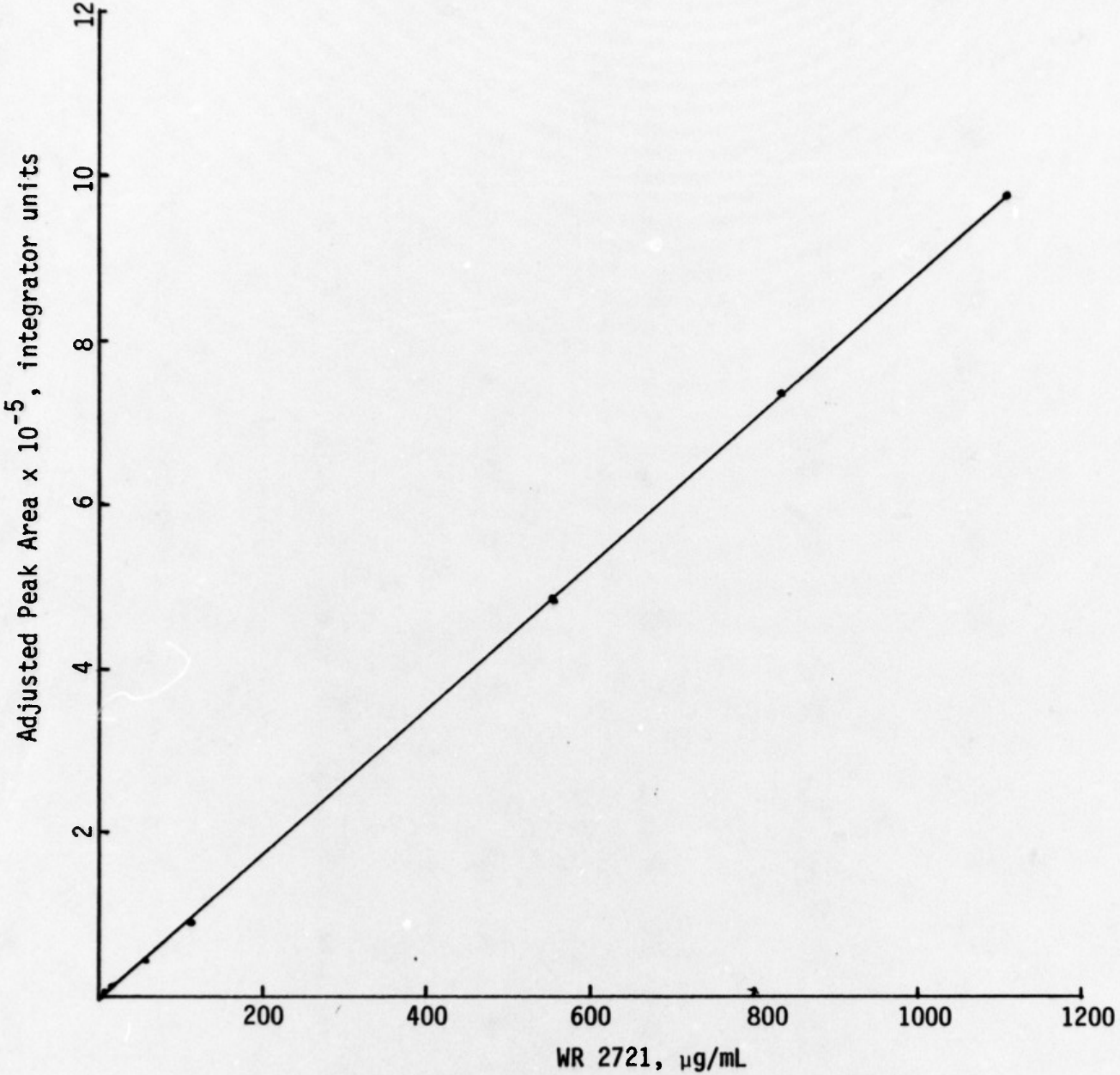


Figure 3. Standard Curve. Samples of dog plasma spiked with WR 2721 and radioactive internal standard were derivatized and chromatographed. Points were corrected for recovery of internal standard.

BIOLOGICAL APPLICATION

Plasma levels of WR 2721 were monitored after two intravenous dosings of a beagle dog. As shown in Figure 4, the experiments gave essentially the same profile; from an initial level of approximately 1000 μg/mL, the drug level approached the assay sensitivity (~2 μg/mL) within 90 minutes. Samples taken after longer periods apparently did not decay to the zero level and work is in progress to increase sensitivity and to determine whether a low level (<1 μg/mL) interference may be present.

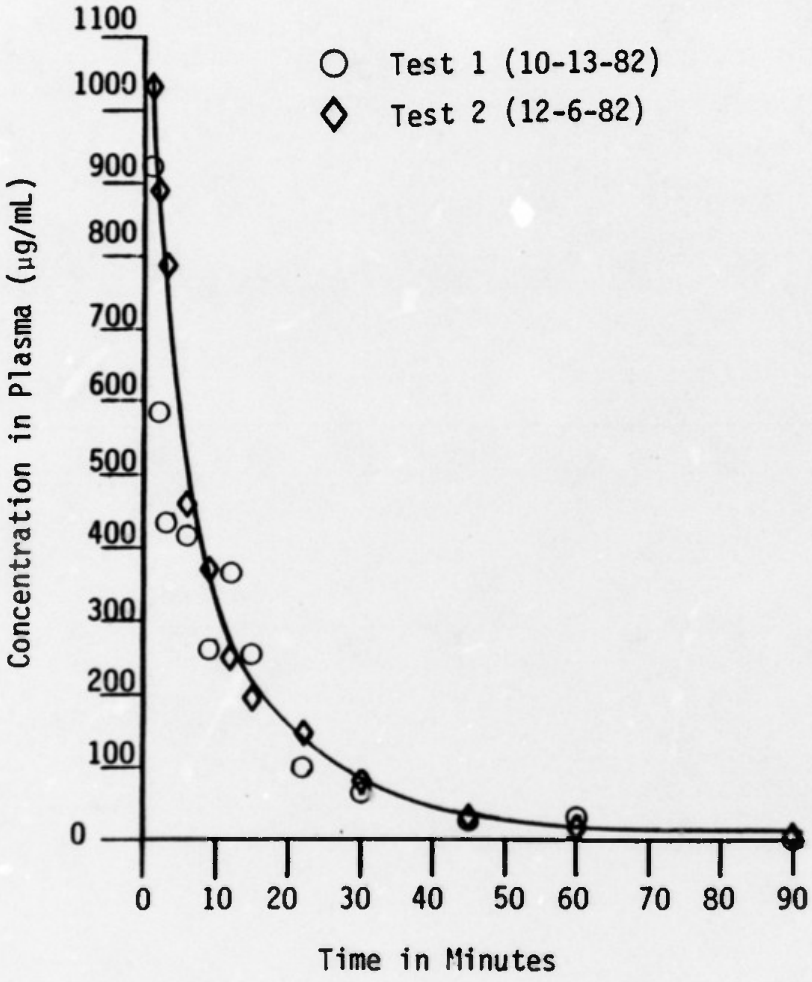


Figure 4. WR 2721 Concentration in Beagle Dog Plasma as a Function of Post Infusion Time.

SUMMARY

A procedure for the analysis of WR 2721 contained in plasma has been developed which requires only a fast derivatization reaction and HPLC separation. The method has been tested in pilot dosing experiments with a beagle dog in which drug levels have been measured from 2- to 1100 µg/mL. A short apparent half-life for the drug was observed in this system. Work is in progress to extend the sensitivity of the method and to allow the use of a nonradiolabeled internal standard.

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8. N. F. Swynnerton, unpublished results.

APPENDIX
SAMPLE CALCULATIONS

Determination of Analyte Recovery

After collection of the blood sample and plasma separation, 90-µL portions of the plasma were transferred to screw capped tubes to be frozen at -70°C and preserved at -20° for future analysis. Prior to freezing, ^{14}C-labeled WR 2721 (16,700 dpm) was added to each plasma portion. After derivatization, 50 µL of the mixture theoretically contained 1193 dpm of ^{14}C-labeled WR 2721. As a check on the addition of the internal standard, a 50-µL portion of each assay mixture was diluted with mobile phase and scintillation fluid and counted. Single operator precision for this addition was very good (mean = 1192, n = 54, SD = 29). Portions (50 µL) of each assay mixture were subjected to high performance liquid chromatographic analysis. Effluent containing ^{14}C-labeled WR 2721 was collected, diluted with scintillation fluid and counted. Total dpm recovered in the column eluant in the retention volume window of WR 2721 divided by the amount measured in the 50-µL assay volume prior to anaysis gives the fraction recovered (recovery factor, see below).

Calculation of WR 2721 Concentration in Plasma

WR 2721 concentration in plasma was calculated using equations (1) and (2).

$$C_S = A_S \times \frac{1}{G} \times \frac{1}{R} \times F \qquad (1)$$

where:

C_S = concentration of WR 2721 in plasma, µg/mL

A_S = integrated peak area of WR 2721 peak, arbitrary units

G = gain setting of fluorescence detector, dimensionless

R = recovery factor, dpm of collected column effluent/theoretical dpm of injected aliquot

F = detector response factor taken from slope and intercept of a recently-generated standard curve or from a point calibration standard, (µg/mL)/peak area.

The value calculated for C_s includes WR 2721 added to the plasma as an internal standard, therefore the actual WR 2721 concentration is obtained from equation (2).

$$C_a = C_s - C_{std} \qquad (2)$$

where:

C_a = actual concentration of WR 2721 in plasma prior to addition of internal standard, µg/mL

C_{std} = contribution from added ^{14}C-labeled internal standard, µg/mL

Example:

A_s = 90056 area units

G = 16X

R = 589 dpm/1193 dpm

F = (89.7 µg/mL)/85360 area units

C_{std} = 0.776 µg/mL

Combining (1) and (2)

$$C_a = \left(90036 \times \frac{1}{16} \times \frac{1193}{589} \times \frac{89.7}{85360}\right) - 0.776$$

$$= 11.2 \text{ µg/mL}$$

APPENDIX B

HPLC Assay for S-2-(3-Aminopropylamino)ethyl phosphorothioate (WR 2721) in Plasma. Abstracts of Papers, Meeting of the Radiation Research Society, San Antonio, Texas, March 1983.

HPLC Assay for S-2-(3-Aminopropylamino)ethyl phosphorothioate (WR 2721) in Plasma. J. of Liq. Chromatogr., 6:1523-1534, 1983.

(Fb—2) *HPLC Assay for S-2-(3-Aminopropylamino)ethylphosphorothioate (WR 2721) in Plasma.* NOLLIE F. SWYNNERTON,* EDWARD P. MCGOVERN,* DONALD J. MANGOLD,* JOE A. NINO,* EMILY M. GAUSE,* AND LAWRENCE FLECKENSTEIN,* Southwest Research Institute, San Antonio, Texas, 78284, Southwest Foundation for Research and Education, San Antonio, Texas, 78284, and Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D. C., 20012. (Introduced by Melvin H. Heiffer.)

A specific HPLC assay has been developed for determination of the radioprotectant drug WR 2721. The method is based on precolumn derivatization of plasma with fluorescamine, separation with a C-18 cartridge and detection by fluorescence. An external standard was used for calibration, and values were adjusted based upon recovery of added ^{14}C-labeled WR 2721. WR 2721 had a retention time of about 12 minutes using a mobile phase of acetonitrile/water (22:78), 0.01 M in dibutyl ammonium phosphate, at a flow rate of 2 mL/min. Detector response was linear over the range of 8 to 1100 μg/mL, and the sensitivity of the assay was characterized to 3 μg/mL. Precision of the method was tested at 14.2, 92.0, and 892 μg/mL, yielding coefficients of variation of 5.9, 3.6, and 3.2%, respectively. The assay requires 90 μL of plasma and has a total chromatography time of about 45 minutes. 2-(3-Aminopropylamino)ethylthiol (WR 1065) and *bis*-[2-(3-aminopropylamino)ethyl]disulfide (WR 33278), metabolites of the drug, and a variety of primary amines were shown not to interfere with the assay. Suitability of this assay for pharmacokinetic studies was demonstrated in a preliminary experiment in a beagle dog. Following an intravenous dose, plasma drug levels were found to drop rapidly from 1030 μg/mL to the lower limits of assay sensitivity. (Supported by Contract DAMD 17-80-C-0128 with the U. S. Army Medical Research and Development Command, Ft. Detrick, Maryland.)

Abstracts of Papers, Meeting of the Radiation Research Society, San Antonio, Texas, Marsh 1983.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(8), 1523-1534 (1983)

HPLC ASSAY FOR S-2-(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOATE (WR 2721) IN PLASMA

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ABSTRACT

A specific HPLC assay has been developed for determination of the radioprotective drug WR 2721. The method is based on precolumn derivatization of plasma with fluorescamine, separation with a C-18 cartridge and detection by fluorescence. An external standard was used for calibration, and values were adjusted based upon recovery of added ^{14}C-labeled WR 2721. WR 2721 had a retention time of about 13 minutes using a mobile phase of acetonitrile/water (22:78), 0.01 M in dibutylammonium phosphate, at a flow rate of 2 mL/min. Sensitivity of the assay was characterized to 2 µg/mL, and detector response was linear over the range of 2 to 1100 µg/mL. The assay requires 90 µL of plasma and has a total chromatography time of about 45 minutes. 2-(3-Aminopropylamino)ethanethiol (WR 1065) and bis-[2-(3-aminopropylamino)ethyl]disulfide (WR 33278), metabolites of the drug, and a variety of primary amines were shown not to interfere with the assay. Suitability of this assay for pharmacokinetic studies was demonstrated in preliminary experiments with a beagle dog.

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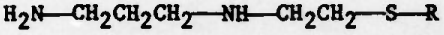
INTRODUCTION

The chemical radioprotector S-2-(3-aminopropylamino)ethyl phosphorothioate (I) has been the object of intense study by research groups of the U.S. Army and the National Cancer Institute. An Army program established in 1959 found that the drug [labeled Walter Reed (WR)-2721] protects various tissues from radiation (1). It was also demonstrated that certain solid animal tumors are not protected, which suggested its use in radiotherapy (2).



I, WR 2721, R = $PO(OH)_2$
II, WR 1065, R = H
III, WR 33278, R = $SCH_2CH_2NHCH_2CH_2CH_2NH_2$

It is believed that in the body the drug is transported intact into the tissue where it is enzymatically cleaved to yield the thiol II (WR 1065) (3). Free radicals produced by radiation are then scavenged by interaction with the sulfhydryl (SH) group of II (4).

A major shortcoming of WR 2721 is that its activity after oral dosing is severely limited--probably due to premature cleavage of the phosphate group under the acidic conditions encountered immediately after oral dosing (5).

Attempted bioassay of WR 2721 has been hampered by several serious obstacles. The compound is acid-labile, has no convenient chromophore, has essentially no solubility in organic solvents and is extremely polar, capable of existing as a dipolar ion or a bis-dipolar ion. Lack of solubility precludes its extraction from biological fluids and its polarity places limits on the types of chromatographic systems which might be used to separate it from endogenous materials. To facilitate

pharmacokinetic studies of the drug, an analytical procedure capable of efficiently processing large numbers of samples is also an important consideration. An HPLC method which overcomes the stated problems and is capable of handling multiple samples is presented in this paper.

MATERIALS AND METHODS

Instrumentation

A Waters Associates Model 244 Liquid Chromatograph equipped with a Model 420 AC Fluorescence Detector and Data Module was employed. Excitation wavelength was 395 nm and emission wavelength was >460 nm. Samples were injected using a Rheodyne Model 7125 Injector fitted with a 20- or 50-μL loop. Separations were carried out on a Waters Aassociates RCM-100 Radial Compression Module fitted with a 100-mm x 8-mm cartridge filled with 5-μm spherical C-18 packing. The analytical column was protected with a Whatman, Inc. guard column filled with Waters Associates CoPell C-18 packing. The mobile phase was acetonitrile/water (22:78), 0.01 M in dibutylammonium phosphate (pH ~3) at a flow of 2.0 mL/min.

Reagents

Acetonitrile was purchased from Burdick and Jackson Laboratories, Inc. Dibutylamine and fluorescamine were obtained from Aldrich Chemical Company. Concentrated (1 M) solutions of dibutylammonium phosphate were prepared by titrating 12.9 g of dibutylamine to pH 2.5 with phosphoric acid and diluting to volume. Fresh solutions containing fluorescamine (5 mg/mL) were prepared weekly using reagent grade acetone which had been stored over 4A molecular sieves. WR 2721 trihydrate, Lot AU-BJ 09506AJ-68-2, was furnished in >99.0% purity by the Walter Reed Army

Institute of Research. Radiolabeled WR 2721, S-[2-(3-aminopropylamino)ethyl-1,2-^{14}C]phosphorothioate was obtained from Research Triangle Institute, Lot 3874-52; its specific activity was 86.0 μCi/mg and reported purity was >97.0%.

Standards containing WR 2721 were prepared by dissolution in 0.05 M sodium borate-potassium chloride pH 10 buffer.

Sample Preparation

Plasma (90 μL), 50 μL of a solution of the radiolabeled internal standard (1.40 μg/mL) and 160 μL of 0.05 M sodium borate-potassium chloride buffer were placed in a polyethylene vial and, while the mixture was being agitated using a vortex mixer (American Scientific Products), 200 μL of the fluorescamine reagent was added. After mixing for 60 s, the mixture was treated with an additional 200 μL of fluorescamine reagent and agitation was continued for 20-30 s. The resulting mixture was centrifuged at 1500 rpm for three minutes and an aliquot of the supernatant was injected onto the HPLC column. Each frozen sample was individually thawed, derivatized and immediately injected because both WR 2721 and its derivative decompose in plasma at room temperature.

Internal Standard

Plasma samples were spiked with increasing amounts of WR 2721, mixed with internal standard, derivatized and injected as described above. The following concentrations were used to construct a standard curve: 0, 1.89, 6.33, 11.9, 56.3, 112. 279, 556, 834, and 1110 μg/mL. The curve was constructed by fitting a regression line to the peak area versus concentration data after correction for recovery. Recovery was determined by adding 50 μL of a solution containing ^{14}C-labeled WR 2721 (activity 334 dpm/μL) to each sample vial prior to derivatization. After

derivatization, a 20- or 50-µL sample was removed from the reaction vial, mixed with scintillation fluid and counted using a Packard Tri-Carb Model 4530 scintillation counter. Similarly, either a 20- or 50-µL sample was analyzed using HPLC. Twelve 1-mL fractions of column effluent were collected starting three minutes prior to the elution time of WR 2721 and continuing for six minutes. Each vial, after addition of scintillation fluid, was counted for 10 minutes, and the recovered radioactivity was determined by summing the disintegrations observed in those fractions with activity greater than the background level. Recovery of WR 2721 was then determined for each assay using the expression: % recovery = (dpm recovered x 100)/dpm added. All calculations were corrected for contributions due to background. Counting efficiencies were determined via automatic external standardization. Recoveries obtained were between 35% and 55% (mean = 48%, n = 58).

Animal Dosing Experiments

A healthy, one-year old, AKC-registerable, male beagle dog weighing 12.7 kg was used in pilot dosing experiments to test the analytical method. The animal was dosed intravenously in the cephalic vein with a 0.9% saline solution containing 1.9 g (150 mg/kg body weight) of WR 2721. The infusion required two minutes. Blood samples (3 mL) were withdrawn into an EDTA Vacutainer® from a cannula placed in the jugular vein. Each sample was immediately chilled in an ice/water bath and then centrifuged. A 90-µL aliquot of the separated plasma was added to 50 µL of the internal standard, the mixture was agitated using a vortex mixer and was then quick-frozen in a dry ice/alcohol bath. Samples were stored at -20°C until time of analysis when they were thawed at room temperature and immediately treated as described in the Sample Preparation section. The drug was shown

to be stable in plasma up to about 30 days when stored at -20°C or lower, but was unstable at room temperature.

RESULTS AND DISCUSSION

Derivatization

Major obstacles in the development of the assay were the instability of WR 2721 in acidic media and its lack of a suitable chromophore. A pH-hydrolysis rate profile (Figure 1) demonstrates the lack of stability under acidic conditions. The data were generated by measuring the rate of appearance of WR 1065 (6) and calculating the rate of disappearance of WR 2721.

In simple aqueous systems underivatized WR 2721 has been analyzed by HPLC using separation on a Whatman PAC column (7) and UV detection at 204 nm, but this procedure was unsuitable for plasma analysis. Derivatization of the drug with fluorescamine allowed detection in the picomole range and at the same time modified its chromatographic behavior such that it was retained on reverse-phase columns.

It was found that precision was remarkably improved by a two-stage derivatization procedure (see Methods section). This may be attributed to the effect of the acetone solvent which, by protein denaturization, could release bound material. Pretreatment of plasma with methanol, ethanol or acetonitrile did not provide a satisfactory increase in precision.

Derivatization with o-phthalaldehyde was briefly investigated but was not further pursued when the derivative was found to be very unstable.

Separation

Figure 2 illustrates a typical chromatogram for a plasma sample spiked with WR 2721 at the 5 µg/mL level. Note that the

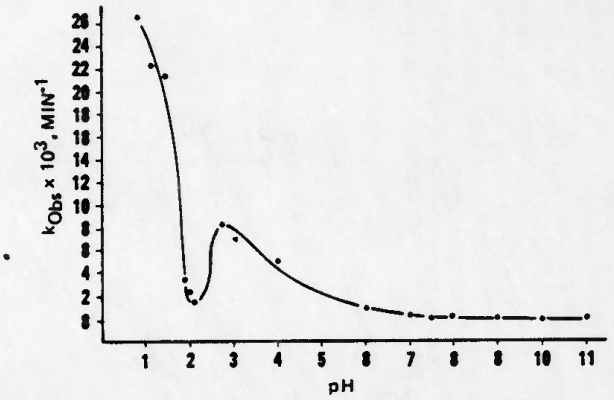


Figure 1. pH-Rate Profile for Hydrolysis of WR 2721 at 37°C

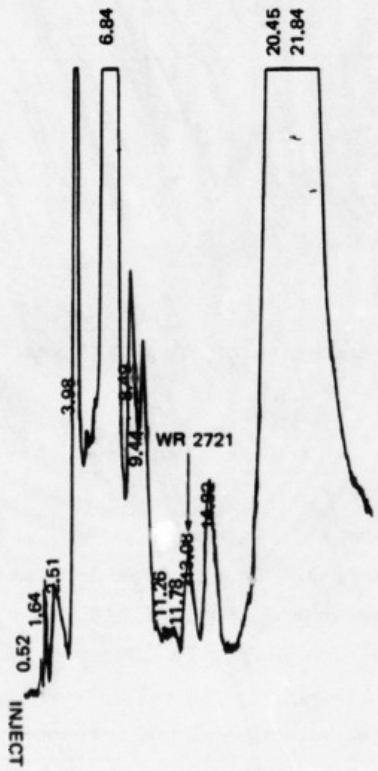


Figure 2. Chromatogram of Beagle Plasma Containing WR 2721 (5 µg/mL)

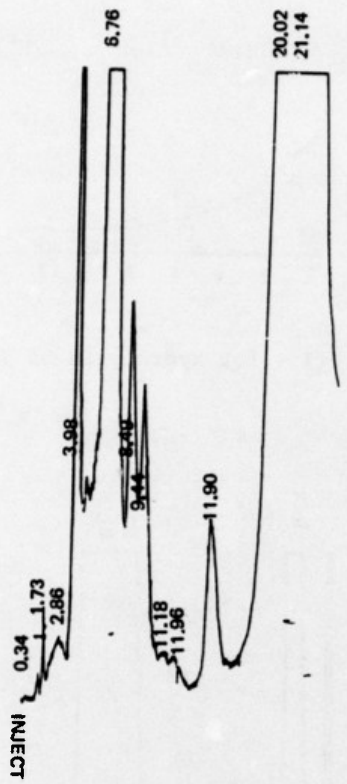


Figure 3. Chromatogram of Beagle Plasma (Blank)

peak of interest was well separated from those peaks due to endogenous materials, thought to be amino acids. A chromatogram of a plasma blank is shown in Figure 3.

The separation was performed under isocratic conditions to increase sample throughput and increase precision. As a result, less polar materials (including WR 1065 and WR 33278) were not eluted and, when allowed to remain, seriously degraded resolution. Flushing the column with methanol/water (70:30) at the end of each analysis day restored column resolution and increased its lifetime.

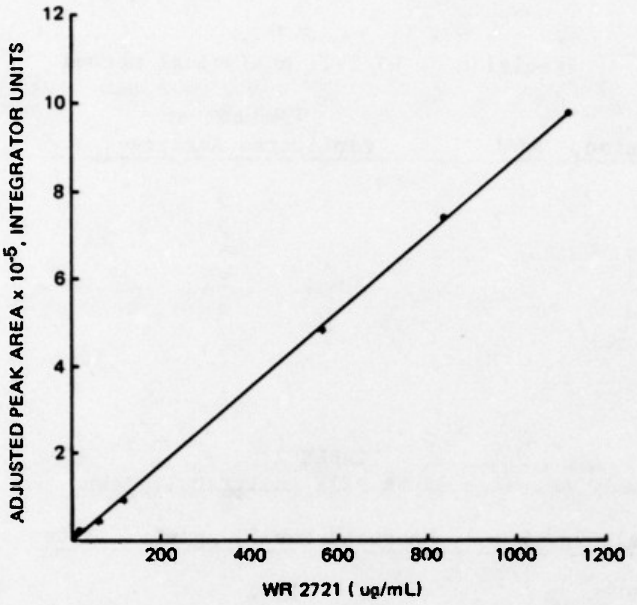


Figure 4. Standard Curve. Samples of beagle plasma spiked with WR 2721 and internal standard were derivatized and chromatographed. Points were corrected for recovery of internal standard.

Internal Standard

As can be seen in Figure 2, the complex chromatogram of derivatized plasma did not offer much hope of finding a suitable internal standard, i.e., one which would have similar behavior toward hydrolysis during sample storage and workup, and elute in a reasonable time in an unobstructed region of the chromatogram. A number of homologs of WR 2721, several other phosphorothioates and a number of amino acids were screened without success. Therefore, radiolabeled WR 2721 was added to plasma samples immediately after they were drawn and the column effluent was collected and counted to determine recovery. Excellent precision and accuracy values were obtained over the approximate range of 2 to 1100 μg/mL using this procedure.

TABLE 1
Precision of WR 2721 Analytical Method

WR 2721 Concentration, g/mL	Number of Replicates Analyzed, n	RSD (%)
1110	5	9.1
112	5	5.3
11.9	5	5.6
6.3	5	5.1
3.0	5	7.8
	Average =	6.6

TABLE 2
Accuracy of WR 2721 Analytical Method

Spike Level, g/mL	Measured Level, g/mL	% Deviation (D)
1.89	1.92	1.6
8.55	8.75	2.3
34.1	33.1	-3.0
78.6	75.9	-3.4
112	107	4.5
390	430	10.2
779	742	-4.8
1110	1050	-5.4

$$\text{Average Deviation} = \frac{|D|}{n} = 4.4$$

Linearity

Figure 4 depicts the relationship between the drug plasma levels and the peak area for derivatized WR 2721. Linear regression of peak area versus concentration gave a coefficient of determination (R^2) of 0.9969, with slope of 930 and intercept of -7684.

Precision and Accuracy

Precision of the method over the entire working range was determined by the analysis of replicate spiked samples. In

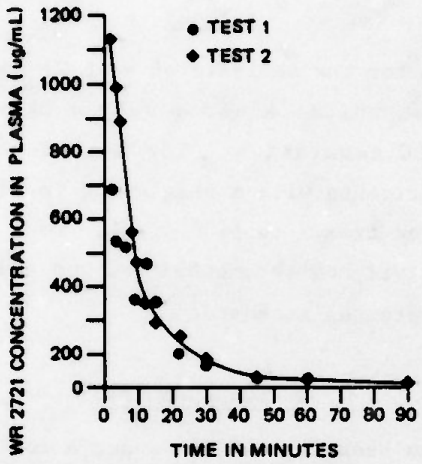


Figure 5. WR 2721 Concentration in Beagle Plasma as a Function of Post-Infusion Time

Table 1 the concentration, number of replicates analyzed and the relative standard deviation (RSD) for each data set is presented. Average RSD for the method was 6.6%.

Accuracy of the method for plasma concentrations ranging from 1.89 to 1110 g/mL was determined by the analysis of blind spiked plasma samples. Average deviation for eight determinations was 4.4%. Spike levels and measured levels are presented in Table 2.

BIOLOGICAL APPLICATION

Plasma levels of WR 2721 were monitored after two intravenous dosings of a beagle dog. As shown in Figure 5, the experiments gave essentially the same profile; from an initial level of approximately 1000 µg/mL, the drug level approached the assay sensitivity (~2 µg/mL) within 90 minutes. Samples taken after longer periods did not decay to the zero level and work is in progress to increase sensitivity and to determine whether a low level (<1 µg/mL) interference was present.

SUMMARY

A procedure for the analysis of WR 2721 contained in plasma has been developed which requires only a fast derivatization reaction and HPLC separation. The method has been tested in pilot dosing experiments with a beagle dog in which drug levels have been measured from 2 to 1100 μg/mL. Work is in progress to extend the sensitivity of the method and to allow the use of a nonradiolabeled internal standard.

ACKNOWLEDGEMENT

This work has been carried out under a contract (DAMD 17-80-C-0128) with the Walter Reed Army Institute of Research as part of a program to develop a protective coating for WR 2721.

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APPENDIX C

Interim Report No. 3
An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl
Phosphorothioate (WR 2721) in Plasma

AD _____

Interim Report 3

An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma

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Nollie F. Swynnerton, Ph.D., Senior Research Scientist
Edward P. McGovern, Ph.D., Senior Research Scientist
Joe A. Nino, Senior Technician

December 1983

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report contains a description of an analytical procedure for the determination of WR 2721 in plasma.

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Interim Report 3

An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma

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SUMMARY

An HPLC assay has been developed for the detection and quantitation of the radioprotective drug S-2-(3-aminopropylamino)ethyl phosphorothioate (WR 2721, ethiofos) present in plasma. Using precolumn derivatization with fluorescamine and S-3-(4-aminobutylamino)propyl phosphorothioate (WR 80855, a homolog of WR 2721) as an internal standard, drug levels of 0.05 to 1000 µg/mL were determined with excellent precision (CV = <5% over the concentration range). Accuracy of the method was 5.5%. An isocratic mobile phase of acetonitrile/ethanol/water (16:7:77) modified with 0.01 M tetrabutylammonium phosphate eluted the drug in 23 minutes and the internal standard in 26 minutes from the C-18 reverse phase column. Detector response was linear over the entire range. The assay uses 150 µL of plasma and requires a total chromatography time of about 50 minutes. Suitability for pharmacokinetic studies was demonstrated in two preliminary experiments with beagle dogs. No interferences due to plasma constituents or drug metabolites were observed.

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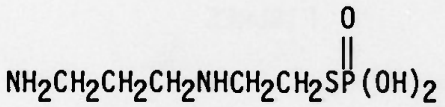
I. INTRODUCTION

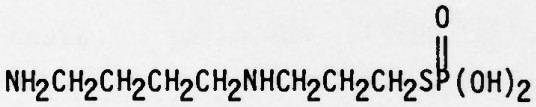
More than 20 years have passed since the finding[1] that S-2-(3-aminopropylamino)ethyl phosphorothioate (WR 2721, I) protected various tissues from radiation. Work continues to determine the efficacy of this versatile drug in a variety of biological applications.

We previously reported an HPLC assay for the drug when present in the plasma of laboratory animals.[2] The method was shown to be accurate and precise over an extended range of 2 to 1100 µg/mL and to require less than one hour for a complete analysis. Radiolabeled WR 2721 was added to determine drug recovery after sample storage and preparation for analysis. Use of this standard placed a limit on detectability and tended to reduce analytical precision at the lowest drug levels.

The objectives of this work were 1) to find a suitable replacement for the radiolabeled WR 2721 internal standard and 2) reduce the lower limit of detectability of WR 2721 to 0.1 µg/mL or less. This paper details the modifications which accomplished the objectives, provides the precision and accuracy estimates of the method and describes its use in the analysis of plasma samples taken from test animals following intravenous dosing of the drug.



I, WR 2721



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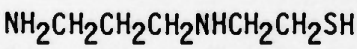
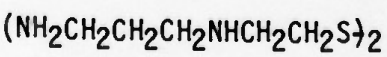


III, WR 1065



IV, WR 33278

II. MATERIALS AND METHODS

A. Instrumentation

A Waters Associates Model 244 Liquid Chromatograph equipped with a Laboratory Data Control Fluoromonitor III Fluorescence Detector and a Waters Associates Data Module was employed. Detector excitation source was a 340-380 nm low pressure mercury and phosphor conversion lamp. A 370 nm bandpass filter was used for excitation and a 418-700 nm cutoff filter was used for emission. Samples were injected using a Rheodyne Model 7125 Injector fitted with a 50-µL loop. Separations were carried out on a Waters Associates RCM-100 Radial Compression Module fitted with a 100-mm x 8-mm cartridge filled with 5-µm spherical C-18 packing. The analytical column was protected by a Whatman, Inc. guard column filled with Waters Associates CoPell C-18 packing. Mobile phases were either acetonitrile/water (22:78) or acetonitrile/ethanol/water (16:7:77), both modified with tetrabutylammonium phosphate (TBAP, 0.01 M, pH ~3) at a flow of 2.0 mL/min.

B. Reagents

HPLC-grade acetonitrile was purchased from J. T. Baker Chemical Company and USP absolute ethanol from U.S. Industrial Chemicals Company. Tetrabutylammonium hydroxide, 40 weight percent solution in water and fluorescamine were obtained from Aldrich Chemical Company. Concentrated (1 M) solutions of tetrabutylammonium phosphate were prepared by titrating 65 mL of 40% tetrabutylammonium hydroxide solution to pH 2.5 with phosphoric acid and diluting to volume. Fresh solutions containing fluorescamine (5 mg/mL) were prepared weekly using reagent grade acetone which had been stored over 4A molecular sieves. WR 2721 trihydrate, Lot AX, BK02762,PB-V-116 and WR 80855, Lot AG49364 were furnished in >99.0% purity by the Walter Reed Army Institute of Research which also supplied reference samples of WR 1065 and WR 33278.

Standards containing WR 2721 and WR 80855 were prepared by dissolution in 0.05 M sodium borate-potassium chloride pH 10 buffer.

C. Sample Preparation

Plasma (150 µL), 150 µL of WR 80855 standard solution and 200 µL of 0.05 M sodium borate-potassium chloride buffer (pH 7.6) were placed in a polyethylene vial and while the mixture was being agitated with a vortex mixer (American Scientific Products) 250 µL of the fluorescamine reagent was added. After mixing for 60 seconds, the mixture was treated with an additional 250 µL of fluorescamine reagent and agitation was continued for 20-30 seconds. The resulting mixture was centrifuged at 1500 rpm for three minutes and an aliquot of the supernatant was injected onto the HPLC column. It has recently become standard practice to filter the supernatant through a 0.45 µm filter prior to injection.

Alternatively, prior to derivatization the plasma containing the added internal standard was quick-frozen in a Dry Ice/isopropyl alcohol bath and stored in a styrofoam box containing Dry Ice until time of analysis. Each frozen sample was then individually thawed and treated as described above.

D. Animal Dosing Experiments

The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards as set forth by the "Guide for the Care and Use of Laboratory Animals" DHEW Publication, NIH 74-23.

Healthy male beagle dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, Michigan, and used in pilot dosing experiments to test the analytical method. The first test was conducted October 4, 1983 and used a 1-year old animal weighing 15.4 kg. The dog was dosed intravenously in the cephalic vein with a 0.9% saline solution containing 2.32 g (150 mg/kg body weight) of WR 2721. The infusion required 2 minutes. The second dosing was carried out October 26, 1983 with a 9-month old dog weighing 13.6 kg. This animal was dosed intravenously in the cephalic vein with a 0.9% saline solution containing 2.05 g (150 mg/kg body weight) of WR 2721 but the infusion time was 10 minutes. Blood samples (3 mL) were withdrawn into an EDTA Vacutainer from a cannula placed in the jugular vein. Each sample was immediately chilled in an ice/water bath and then centrifuged. A 150-µL aliquot of the separated plasma was added to 150 µL of the internal standard, the mixture was agitated using a Vortex mixer and then quick-frozen in a Dry Ice/isopropyl alcohol bath. Samples were stored at -78°C until time of analysis when they were thawed at room temperature and immediately treated as described in the Sample Preparation section.

III. RESULTS AND DISCUSSION

A. Internal Standard

Among a number of non-radiolabeled candidates investigated as potential internal standards for the HPLC assay of WR 2721 (I), a homolog, S-3-(4-aminobutylamino)propyl phosphorothioate (WR 80855, II) was found to have essentially the same chemical behavior. Its derivatization with fluorescamine was rapid and repeatable, and similar short-term stabilities were observed for I and II in basic buffer solutions.

B. Sensitivity

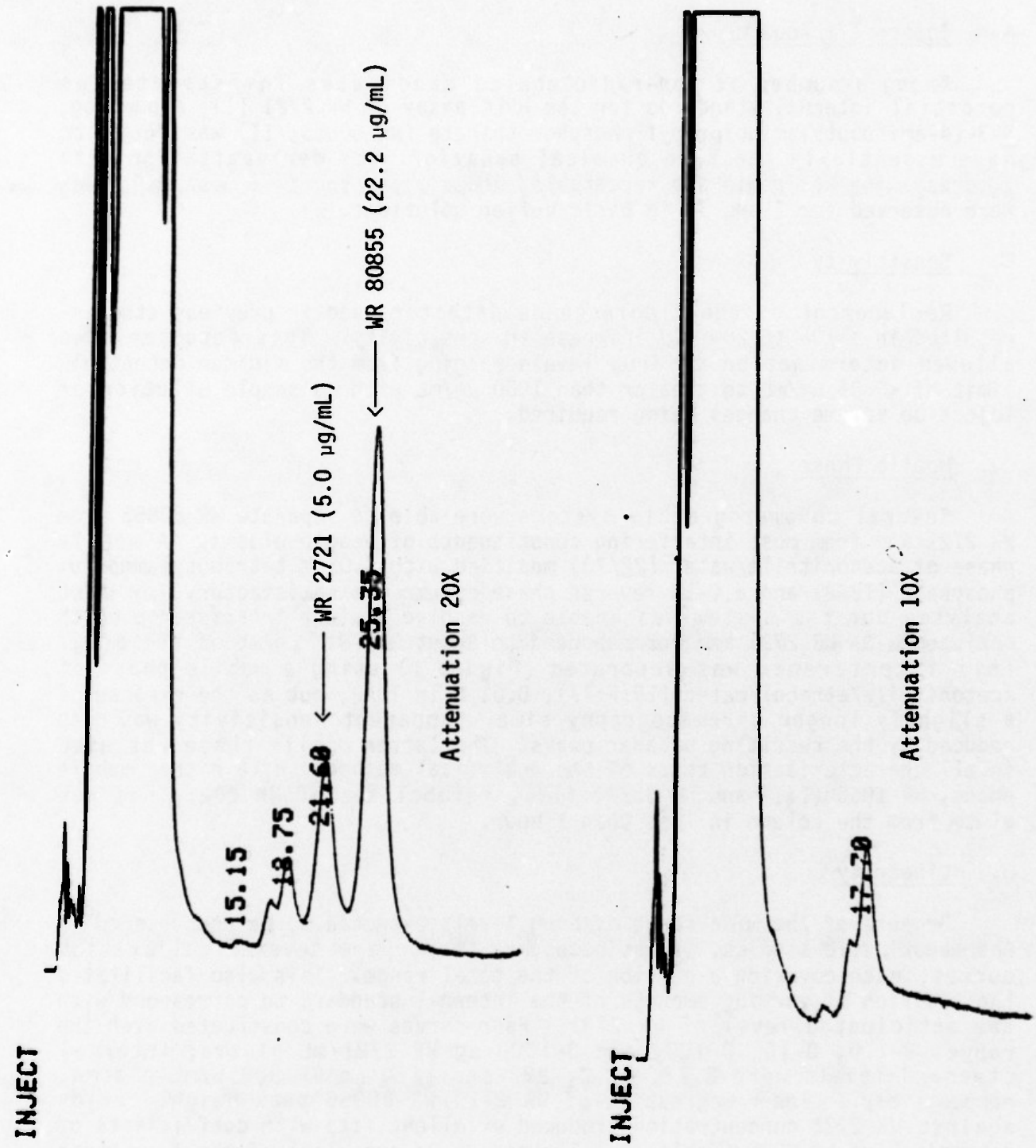
Replacement of the fluorescence detector used in previous studies[2] resulted in a 10- to 20-fold increase in sensitivity. This detector also allowed determination of drug levels ranging from the minimum detectable limit of ~0.05 µg/mL to greater than 1000 µg/mL with no sample dilution or injection volume changes being required.

C. Mobile Phase

Several chromatographic systems were able to separate WR 80855 from WR 2721 and from most interfering constituents of beagle plasma. A mobile phase of acetonitrile/water (22/78) modified with 0.01 M tetrabutylammonium phosphate (TBAP) and a C-18 reverse phase column was satisfactory for most analyses but the system was unable to resolve a minor interference which coeluted with WR 2721 and corresponded to about 0.2-0.3 µg/mL of the drug. This interference was separated (Figure 1) using a mobile phase of acetonitrile/ethanol/water (16:7:77), 0.01 M in TBAP, but at the expense of a slightly longer chromatography time. Apparent sensitivity was also reduced by the resulting broader peaks. The latter mobile phase was used in all characterization tests of the analytical method. With either mobile phase, WR 1065 (III) and WR 33279 (IV), metabolites of WR 2721, did not elute from the column in less than 1 hour.

D. Linearity

Because of the wide range of drug levels expected to be encountered in pharmacokinetic studies, it was necessary to prepare several calibration curves, each covering a portion of the total range. This also facilitated the addition of varying amounts of the internal standard to correspond with the anticipated level of WR 2721. Four curves were constructed over the ranges 0-1.0, 0-10, 0-100, and 0-1000 µg WR 2721/mL plasma; internal standard levels were 2.22, 22.2, 222 and 1110 µg WR 80855/mL plasma, respectively. Linear regression of WR 2721/WR 80855 peak height ratios against WR 2721 concentration produced excellent fits with coefficients of determination $\geq$0.9986 (Table 1). Figures 2a-2d are plots of the four data sets. In general, use of integrated peak areas gave more scatter in the data than peak height measurements and occasionally gave anomolous values.



a) Beagle plasma spiked with WR 2721 and WR 80855.

b) Beagle plasma (blank).

Figure 1. Representative chromatograms showing separation of fluorescamine derivatives WR 2721 and WR 80855 in beagle plasma.

TABLE 1. STATISTICS OF WR 2721 STANDARD CURVES

	Plasma Levels of WR 2721, µg/mL			
	0-1.0	0-10.0	0-100	0-1000
Concentration of Internal Standard,[a] µg/mL	2.22	22.2	222	1110
Slope of Standard Curve[b]	0.722	0.0678	0.0102	0.00205
Intercept " "	-0.016	-0.00756	0.00428	0.00990
Coefficient of Determination	0.9986	0.9997	1.0000	0.9999
Degrees of Freedom	7	7	6	6

a. S-3-(4-aminobutylamino)propyl phosphorothioate, WR 80855.

b. Linear regression of (peak height of WR 2721)/(peak height of WR 80855) against concentration of WR 2721 in µg/mL.

Figure 2a.

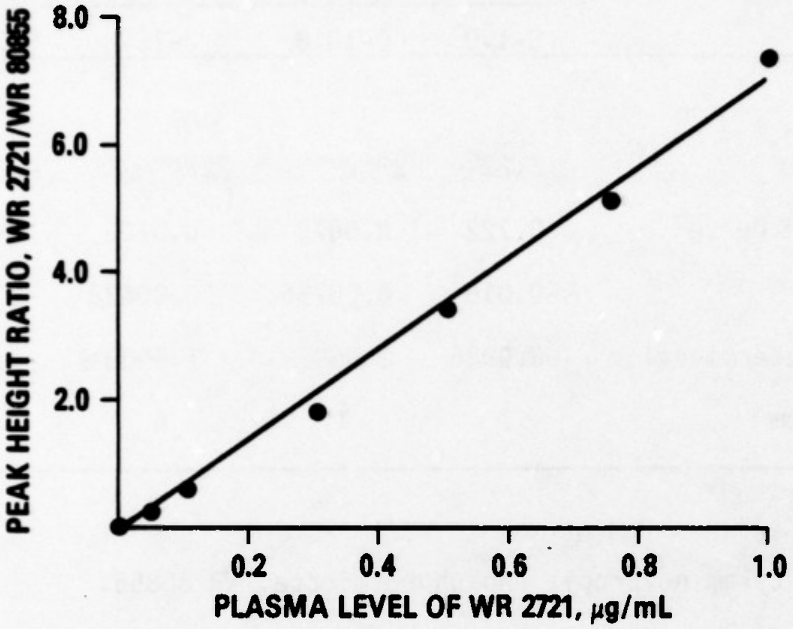


Figure 2b.

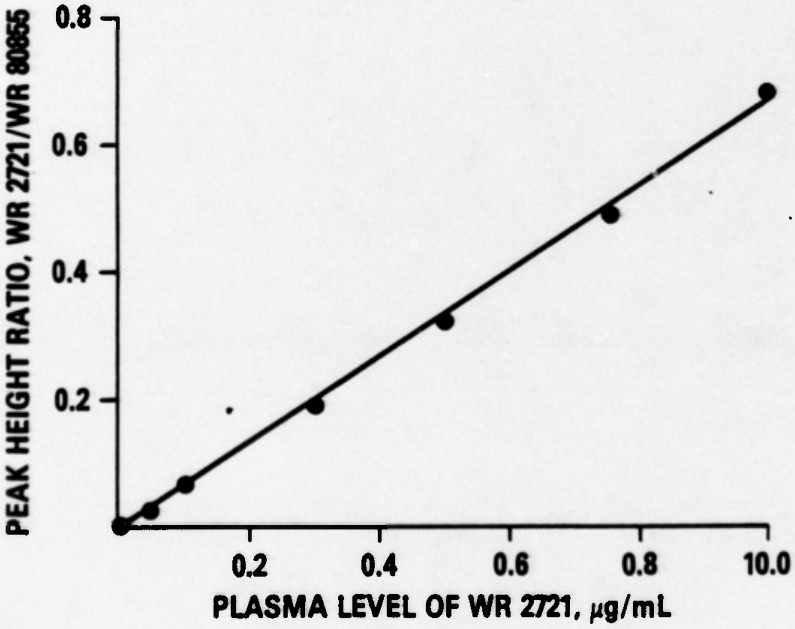


Figure 2a,b. Standard Curves. Samples of beagle plasma spiked with WR 2721 and WR 80855 as Internal Standard.

Figure 2c.

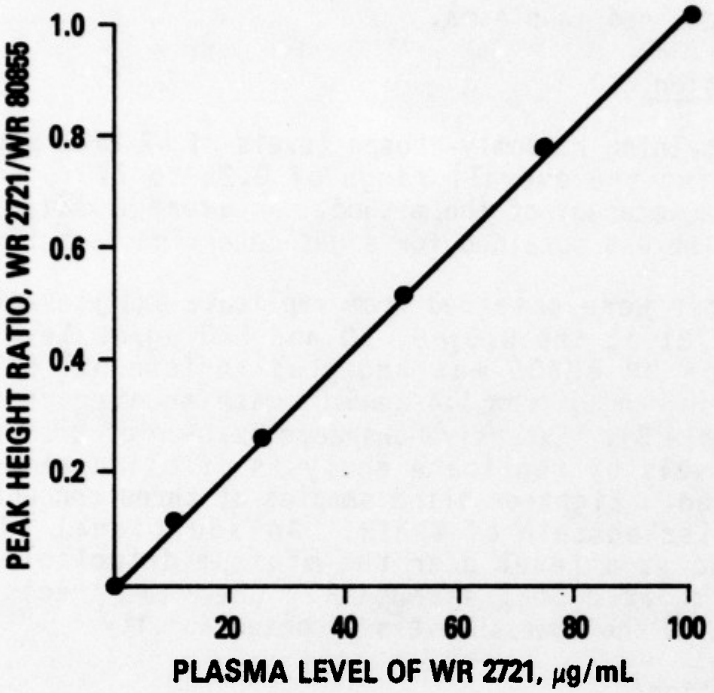


Figure 2d.

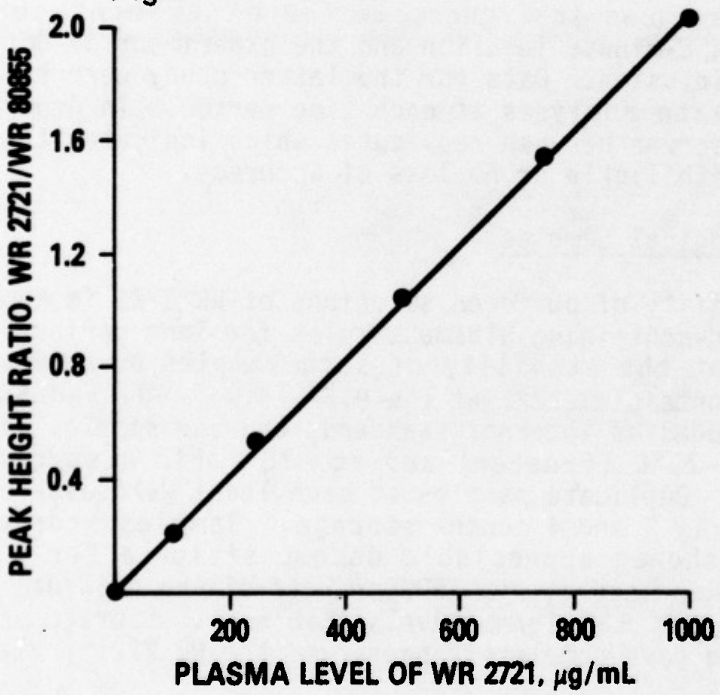


Figure 2c,d. Standard Curves. Samples of beagle plasma spiked with WR 2721 and WR 80855 as Internal Standard.

Standard curves constructed from the analysis of known amounts of WR 2721 in buffer solution were superimposable upon those obtained from analysis of the drug contained in plasma.

E. Accuracy and Precision

Plasma samples containing randomly-chosen levels of WR 2721 within the four ranges and spanning the overall range of 0.25 to 770 µg/mL were assayed to determine the accuracy of the method. An average deviation of 5.5% from the actual value was obtained for eight determinations (Table 2).

Precision estimates were obtained from replicate analyses of beagle plasma spiked with WR 2721 at the 0.5, 5, 50 and 500 µg/mL levels. An appropriate amount of WR 80855 was added as an internal standard. Coefficients of variation ranged from 1.4 to 5.0% with an average of 2.9% for the four levels (Table 3). Extensive characterization of the method at the lower detection levels by replicate analysis of blind samples has recently been completed. Eighteen blind samples at three concentrations were prepared by L. Fleckenstein of WRAIR. An additional six were independently prepared at a level near the minimum detectable limit. Results (Table 4) demonstrated that essentially the same precision and accuracy was attainable at the lowest levels of detectability.

F. Biological Application

Time course of plasma levels of WR 2721 following intravenous dosing of two beagle dogs is shown in Figure 3. The experiment conducted October 4, 1983 used a 2-minute infusion and the experiment of October 26, 1983 used a 10-minute infusion. Data for the latter study were the average of values from duplicate analyses at each time period. In general, very close agreement was observed between replicates which indicates that single analyses may be used with little or no loss of accuracy.

G. Stability of Biological Samples

Although the stability of buffered solutions of WR 2721 is known[2], the necessity to store drug-containing plasma samples for long periods prior to analysis required that the stability of such samples be known. Beagle plasma was spiked to contain WR 2721 at the 0.5-, 5.0-, 50- and 500-µg/mL levels, WR 80855 was added as internal standard, and the samples were quick frozen and stored at -20°C (freezer) and at -78°C (in a styrofoam box containing Dry Ice). Duplicate samples at each level were assayed by the described method after 1, 2 and 4 months storage. Samples stored at the higher temperature showed appreciable decomposition after 2 months, particularly at the lower levels, with 50% or less of the original WR 2721 remaining of the 0.5 and 5.0 µg/mL levels (Table 5). Storage at Dry Ice temperature appeared to have completely preserved the WR 2721 (relative to WR 80855) for 2 months.

TABLE 2. ACCURACY OF WR 2721 ANALYTICAL METHOD

Spike Level, µg/mL	Measured Level, µg/mL	Percent Deviation (D)
0.25	0.27	8.0
0.75	0.72	-4.0
8.0	7.7	-3.8
15.0	16.4	9.3
40.0	36.3	-9.2
90.0	83.0	-7.8
320	315	-1.6
770	765	-0.6

Average Deviation = $\frac{|D|}{n}$ = 5.5%

TABLE 3. PRECISION ESTIMATES OF WR 2721 ASSAY USING WR 80855 AS INTERNAL STANDARD[a]

WR 2721 Plasma Level, µg/mL	0.5	5.0	50	500
Number of Replicates	7	7	5	6
Average peak height ratio	0.576	0.258	0.516	1.06
Standard deviation	0.0206	0.0116	0.00734	0.018
CV, %	3.6	5.0	1.4	1.7

a. Mobile phase of CH_3CN/EtOH/water, 0.01 M in TBAP, 2.0 mL/min.

TABLE 4. ANALYSIS OF BLIND SAMPLES

	Assays WR 2721 Level, µg/mL			
	Level 1	Level 2	Level 3	Level 4
	0.104	0.51	1.1	6.9
	0.096	0.50	1.2	7.4
	0.096	0.51	1.2	7.4
	0.095	0.52	1.2	7.7
	0.087	0.51	1.2	7.6
	0.077	0.57	1.2	
Mean	0.092	0.520	1.18	7.40
Standard Deviation	0.00931	0.0253	0.0408	0.308
CV	10.1%	4.9%	3.4%	4.2%
True Value	0.090	0.54[a]	1.10[a]	7.50[a]
Recovery	102.8%	96.3%	107.0%	98.7%

a. Values of blind samples prepared and reported by L. Fleckenstein. A single blank was included which gave no value for WR 2721, demonstrating no interference.

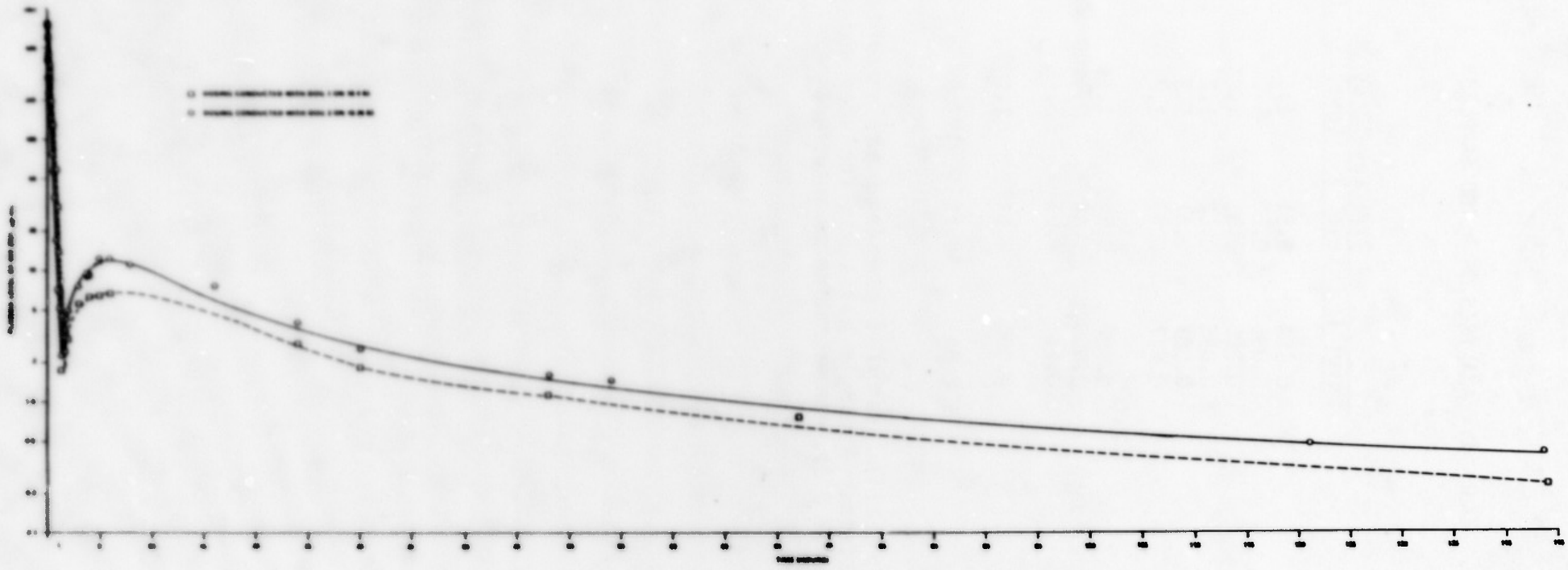


Figure 3. Beagle Plasma Level of WR 2721 Following Intravenous Dosing.

TABLE 5. STABILITY OF WR 2721 IN STORED FROZEN PLASMA[a]

Temperature, °C	WR 2721 Level, µg/mL			
	Zero Time[b]	1 Month[c]	2 Months[c]	4 Months[c]
-20	0.50	0.39	0.17	0.08
-78	0.50	-	0.48	0.56
-20	5.0	3.9	2.5	1.2
-78	5.0	5.1	4.0[d]	4.7
-20	50	41	38	31
-78	50	50	48	49
-20	500	440	415	347
-78	500	485	500	523

a. Beagle plasma, spiked with WR 2721 and internal standard, quick frozen and stored at -20°C or -78°C.

b. Calculated from added volume of freshly prepared standard solutions.

c. Values obtained as average of two determinations using HPLC method described herein.

d. Single determination.

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APPENDIX D

An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl phosphorothioate (WR 2721) in Plasma. Abstracts of Papers, Chemical Modifiers of Cancer Treatment Meeting, Banff, Alberta, Canada, November 1983.

An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl phosphorothioate (WR 2721) in Plasma. International Journal of Radiation Oncology Biology Physics, Vol. 10, pp. 1521-1524.

AN IMPROVED HPLC ASSAY FOR S-2-(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOATE (WR 2721) IN PLASMA

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An HPLC assay is presented for the detection and quantitation of the radioprotective drug S-2-(3-aminopropylamino)ethyl phosphorothioate (WR 2721) present in plasma. Improved selectivity and a 40-fold increase in sensitivity have been demonstrated over the method previously reported by this laboratory.* Using precolumn derivatization with fluorescamine and S-3-(4-aminobutylamino)propyl phosphorothioate (WR 80855, a homolog of WR 2721) as internal standard, drug levels of 0.05 to 1000 µg/mL were determined with excellent precision (CV = <5% over the concentration range). An isocratic mobile phase of acetonitrile/ethanol/water (16:7:77) modified with 0.01 M tetrabutylammonium phosphate eluted the drug in 23 min and the internal standard in 26 min from the C-18 reverse phase column. Detector response was linear over the entire range. The assay uses 90 µL of plasma and requires a total chromatography time of about 50 min. The method was found suitable for pharmacokinetic studies in a preliminary experiment with a beagle dog in which no interferences due to plasma constituents or drug metabolites were observed. (Supported by Contract DAMD 17-80-C-0128 with the U.S. Army Medical Research and Development Command, Ft. Detrick, MD).

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*J. Liq. Chromatogr., 6, 1523 (1983).

Abstracts of Papers, Chemical Modifiers of Cancer Treatment Meeting, Banff, Alberta, Canada, November 1983.

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● *Session IV*

AN IMPROVED HPLC ASSAY FOR *S*-2-(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOATE (WR-2721) IN PLASMA

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An HPLC assay is presented for the detection and quantitation of the radioprotective drug *S*-2-(3-aminopropylamino)ethyl phosphorothioate (WR-2721, ethiofos) present in plasma. Improved selectivity and a 40-fold increase in sensitivity have been demonstrated over the method previously reported by this laboratory. Using precolumn derivatization with fluorescamine and *S*-3-(4-aminobutylamino)propyl phosphorothioate (WR-80855, a homolog of WR-2721) as the internal standard, drug levels of 0.05 to 1000 μg/mL were determined with excellent precision (CV ≤ 5% over the concentration range). An isocratic mobile phase of acetonitrile/ethanol/water (16:7:77) modified with 0.01 M tetrabutylammonium phosphate eluted the drug and the internal standard from the C-18 reverse-phase column in 23 minutes and 26 minutes, respectively. Detector response was linear over the entire range. The assay uses 150 μL of plasma and requires a total chromatography time of about 50 minutes. The method was found suitable for pharmacokinetic studies in a preliminary experiment with a beagle dog in which no interferences due to plasma constituents or drug metabolites were observed.

***S*-2-3(Aminopropylamino)ethyl phosphorothioate, WR-2721, *S*-3-(4-aminobutylamino)propyl phosphorothioate, WR-80855, Plasma, Assay, HPLC, Pharmacokinetic.**

INTRODUCTION

Studies to determine the efficacy of the radioprotector *S*-2-(3-aminopropylamino)ethyl phosphorothioate,[1] (WR-2721, ethiofos) have been hampered by the lack of a sensitive, specific assay for the drug when present in plasma. We have previously reported[2] a method of analysis of the drug which allows detection and quantitation down to a lower limit of approximately 2 μg WR-2721 per mL of plasma. This method has been modified to lower its minimum detectable limit to 0.05 μg/mL and the improved assay has been used to follow postinfusion plasma levels in a beagle dog. Preliminary results of a study of the stability of WR-2721 in stored plasma samples have been obtained using the method and are reported herein.

METHODS AND MATERIALS

Instrumentation

A Waters Model 244 Liquid Chromatograph* equipped with an LDC Fluoromonitor III Fluorescence Detector† and a Waters Data Module* was employed. Detector excitation source was a 340–380 nm low-pressure mercury and phosphor conversion lamp. A 370 nm bandpass filter was used for excitation and a 418–700 nm cutoff filter was used for emission. Samples were injected using a Rheodyne Model 7125 Injector‡ fitted with a 50-μL loop. Separations were carried out on a Waters RCM-100 Radial Compression Module* fitted with a 100-mm × 8-mm cartridge filled with 5-μm spherical C-18 packing. The analytical column was protected by a Whatman guard column§ filled with Waters CoPell C-18 packing.* Mobile phases were either acetonitrile/water (22:78) or acetonitrile/ethanol/water (16:7:77), both modified with tetrabutylammonium phosphate (TBAP, 0.01 M, pH ~3), at a flow of 2.0 mL/min.

Reagents

HPLC-grade acetonitrile was purchased from J. T. Baker Chemical Company** and USP absolute ethanol from U.S. Industrial Chemicals Company.†† Tetrabutylammonium hydroxide, 40 weight percent solution in

Supported by Contract DAMD17-80-C-0128 with the U.S. Army Medical Research and Development Command, Ft. Detrick, MD.

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Accepted for publication 24 April 1984.

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** J. T. Baker Chemical Co., Phillipsburg, NJ.

†† U.S. Industrial Chemicals Company, Tuscola, IL.

water, and fluorescamine were obtained from Aldrich Chemical Company.‡‡ Concentrated (1 M) solutions of tetrabutylammonium phosphate were prepared by titrating 65 mL of 40% tetrabutylammonium hydroxide solution to pH 2.5 with phosphoric acid and diluting to volume. Fresh solutions containing fluorescamine (5 mg/mL) were prepared weekly using reagent-grade acetone which had been stored over 4Å molecular sieves. WR-2721 trihydrate, Lot AX, BK02762, PB-V-116 and WR 80855, Lot AG49364 were furnished in >99.0% purity by the Walter Reed Army Institute of Research, which also supplied reference samples of WR-1065 and WR-33278.

Standards containing WR-2721 and WR-80855 were prepared by dissolution in 0.05 M sodium borate-potassium chloride pH 10 buffer.

Sample preparation

Plasma (150 μL), 150 μL of WR 80855 standard solution and 200 μL of 0.05 M sodium borate-potassium chloride buffer (pH 7.6) were placed in a polyethylene vial and while the mixture was being agitated with a vortex mixer,* 250 μL of the fluorescamine reagent were added. After mixing for 60 seconds, the mixture was treated with an additional 250 μL of fluorescamine reagent and agitation was continued for 20 to 30 seconds. The resulting mixture was centrifuged at 1500 rpm for 3 minutes and an aliquot of the supernatant was injected onto the HPLC column. It has recently become standard practice to filter the supernatant through a 0.45 μm filter prior to injection.

Alternatively, prior to derivatization the plasma containing the added internal standard was quick-frozen in a Dry Ice/isopropyl alcohol bath and stored in a styrofoam box containing Dry Ice until time of analysis. Each frozen sample was then individually thawed and treated as described above.

Animal dosing experiments

The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards as set forth by the "Guide for the Care and Use of Laboratory Animals" DHEW Publication, NIH 74-23.

A healthy, 1 year-old male beagle dog weighing 15.4 kg was purchased from Laboratory Research Enterprises, Inc.† and used in pilot dosing experiments to test the analytical method. The animal was dosed intravenously over 2 minutes in the cephalic vein with a 0.9% saline solution containing 2.32 g (150 mg/kg body weight) of WR-2721. Blood samples (3 mL) were withdrawn into an EDTA Vacutainer‡ from a cannula placed in the jugular vein. Each sample was immediately chilled in an ice/water bath and then centrifuged. A 150-μL aliquot of the separated plasma was added to 150 μL of the internal standard, then the mixture was agitated using a vortex mixer and quick-frozen in a Dry Ice/isopropyl alcohol bath. Samples were stored at −78°C until time of analysis when they were thawed at room temperature and immediately treated as described in the Sample Preparation section.

RESULTS AND DISCUSSION

A homolog of WR-2721, *S*-3-(4-aminobutylamino)propyl phosphorothioate (WR-80855), was chosen as an internal standard based upon its chromatographic behavior and its similar chemical properties. Chromatographic conditions developed for the assay were shown to provide baseline separation between WR-2721 and the internal standard, and no endogenous interferences were observed when random plasma samples from four test animals were analyzed. Fluorescamine derivatives of the thiol metabolite WR-1065 and its symmetrical disulfide WR-33278 did not elute from the column within 1 hour under these conditions.

When the ratio of detector responses (WR-2721/WR-80855) was plotted as a function of WR-2721 concentration, linear standard curves were obtained over the ranges 0–1, 1–10, 10–100 and 100–1000 μg/mL in beagle plasma and in buffer solution (pH 10). Standard curves constructed from the drug in plasma were superimposable upon those of the drug in buffer solution, implying that the recovery from plama was quantitative (or at least the same in both media).

Estimates of the precision and accuracy of the method were obtained from replicate analyses of blind spiked plasma samples, the levels being chosen randomly within each of the four concentration ranges previously mentioned. The average absolute deviation of eight samples from the true value was 5.5%. Coefficients of variation ranged from 1.4 to 5.0% with an average of 2.9% for the four concentration ranges.

Two preliminary dosing studies have been performed to test the analytical method. The first compared this procedure with an earlier method[2] by separate analyses of aliquots taken from each sample. Although results were similar it was obvious that samples stored at −20°C prior to analysis suffered some decomposition of the WR-2721.

‡‡ Aldrich Chemical Co., Milwaukee, WI.

* American Scientific Products, McGraw Park, IL.

† Laboratory Research Enterprises, Inc., Kalamazoo, Michigan.

‡ A registered trademark of Becton, Dickinson & Co., Paramus, New Jersey.

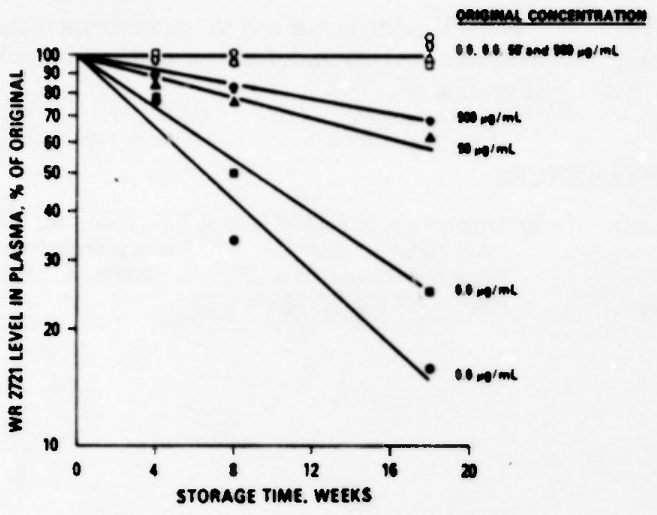

Fig. 1. Stability of WR-2721 in Beagle plasma stored at −20°C (closed symbols) and at −78°C (open symbols).

The extent of decomposition was determined in an experiment in which plasma samples were spiked with WR-2721 at four different levels (0.5, 5, 50 and 500 μg/mL) and stored at either −20°C or −78°C, then analyzed after 1, 2 and 4 months using the method being reported. As can be clearly seen in Figure 1, samples stored at the higher temperature showed appreciable degradation, the extent being dependent upon original drug level. Storage at the lower temperature appeared to protect the drug from decomposition. (It must be noted that since both WR-2721 and internal standard were added to the plasma before storage, any rate of disappearance of WR-2721 is relative to that of WR-80855.)

Protocol of the second dosing study was modified to provide for sample storage at −78°C and to minimize exposure of the samples to any temperature above −78°C. A second change was to modify the selectivity of the HPLC separation to remove a small interference (equal to ~0.3 μg WR-2721/μL plasma). This was done by slightly changing the HPLC mobile phase (see Methods and Materials Section). Results of the dosing are shown plotted in Figure 2. Each point is the result of a single determination using the method reported herein. The improved sensitivity may be seen in the samples taken after about 30 minutes. It was interesting to note that the plasma profile at the shorter postinfusion times was

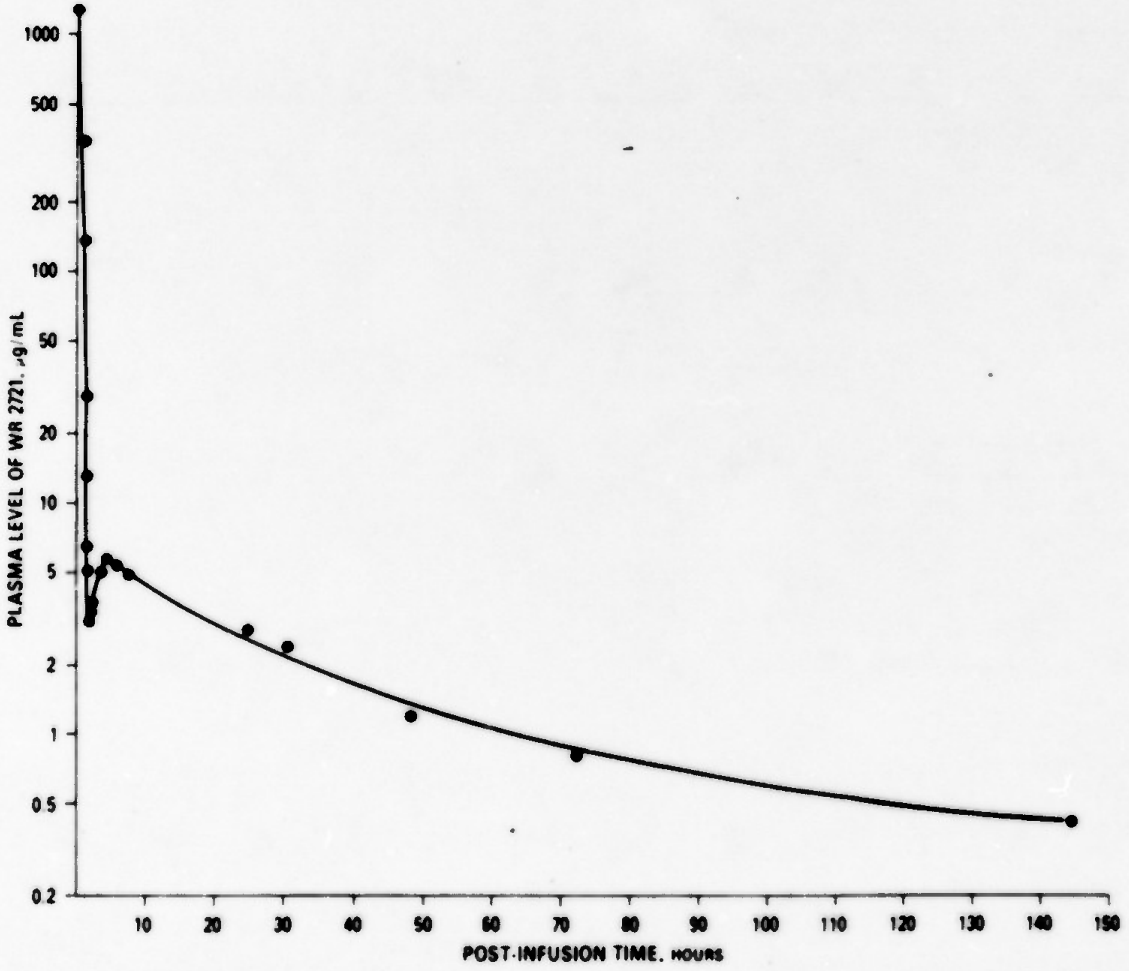

Fig. 2. Beagle plasma level of WR-2721 following intravenous dosing.

similar to those observed in each of the dosing studies we have conducted during the evolution of the present assay.

Work is being continued to improve upon this assay, to shorten analysis time, increase sensitivity and broaden its application.

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APPENDIX E

HPLC Assay for 2-(3-Aminopropylamino)ethanethiol (WR 1065) in Plasma. Abstracts of Papers, Chemical Modifiers of Cancer Treatment Meeting, Banff, Alberta, Canada, November 1983.

HPLC Assay for 2-(3-Aminopropylamino)ethanethiol (WR 1065) in Plasma. International Journal of Radiation Oncology Biology Physics, Vol. 10, pp. 1517-1520.

HPLC ASSAY FOR 2-(3-AMINOPROPYLAMINO)ETHANETHIOL (WR 1065) IN PLASMA

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An HPLC assay for the thiol WR 1065 in plasma is described. WR 1065, a metabolite of the radioprotective drug WR 2721, is separated by HPLC and detected and quantified using an electrochemical cell equipped with a Hg/Au working electrode. No derivitization is required and metabolite plasma levels from 1 µg/mL to 500 µg/mL have been determined with good precision and accuracy as the detector response is linear over the range investigated (R^2 = 0.99). An isocratic mobile phase of 0.1 M aqueous monochloroacetic acid/methanol (85:15) containing 0.005 M camphorsulfonic acid elutes the metabolite from a C-18 reverse phase column in 6 minutes. The assay uses 100 µl of plasma and requires a total chromatography cycle time of 40 minutes. The method has been found suitable for the determination of WR 1065 in plasma from a beagle dog dosed with WR 2721. (Supported by Contract DAMD 17-80-C-0128 with the U.S. Army Medical Research and Development Command, Ft. Detrick, MD).

Abstracts of Papers, Chemical Modifiers of Cancer Treatment Meeting,
Banff, Alberta, Canada, November 1983.

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Session IV

HPLC ASSAY FOR 2-(3-AMINOPROPYLAMINO)ETHANETHIOL (WR-1065) IN PLASMA

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A high pressure liquid chromatography (HPLC) plasma assay for WR-1065 is described which is both precise (coefficient of variation < 5%) and accurate (% average deviation ≤ 6.1) throughout the concentration range from 1 to 500 μg/mL of plasma. The analyte is separated by HPLC and detected with a thiol specific electrochemical transducer cell. The detector response is linear over the ranges 1 to 10 μg/mL (R^2 = 0.995), 10 to 100 μg/mL (R^2 = 0.995), and 100 to 500 μg/mL (R^2 = 0.974). The absolute retention times for WR-1065 and WR-1729 are 9 and 12 minutes, respectively. The assay uses 100 μL of plasma and requires a total chromatography cycle time of 40 minutes. The method has been found suitable for the determination of WR-1065 in plasma from a beagle dog after i.v. administration of S-2-(3-aminopropylamino)ethyl phosphorothioate (WR-2721).

WR-1065, HPLC.

INTRODUCTION

The aminothiol 2 - (3 - aminopropylamino)ethanethiol (WR-1065) formed hydrolytically from the radioprotective drug S-2-(3-aminopropylamino)ethyl phosphorothioate (WR-2721) has been implicated as the compound ultimately responsible for the radioprotection effect.[3,4] To fully understand the protection mechanism and utilize the drug therapeutically most effectively, a rapid, simple, selective and sensitive method for the determination of WR-1065 in biological samples must be available. Several methods for WR-1065 quantitation have been developed;[1,2,4,5,6] however, a rapid, selective and sensitive method for determination of micrograms per milliliter and sub micrograms per milliliter concentrations in biological fluids is still lacking. We shall describe an analytical method based upon HPLC coupled with electrochemical detection, which is rapid (<40 minutes total analysis time), selective (responds to thiols only) and sensitive (quantification at <1 μg/mL plasma possible). A preliminary study involving the analysis of beagle dog plasma samples after dosing with WR-2721 demonstrates the method's applicability and its potential for use in assaying other fluids and tissues as well.

MATERIALS AND METHODS

Instrumentation

A ternary gradient liquid chromatograph* equipped with an amperometric LC detector* and a transducer cell with a Hg/Au working electrode† was employed. The electrochemical cell was operated at a working potential of +0.15 V versus silver/silver chloride. Samples were introduced using an injector‡ equipped with a 20-μL sampling loop. Separations were carried out on a 250 mm × 4.6 mm ID Partisil 5 ODS-3§ prepacked column. The analytical column was protected from strongly adsorbed analytes with a guard column† containing a replaceable 30 mm × 4.6 mm cartridge packed with a 5 μm spherical C-18 packing. The mobile phases were either acetonitrile/0.1 M chloroacetic acid, pH 3.0, 3:97 or 4:96 modified to be 0.005 M in dl-10-camphorsulfonic acid (CSA) at a flow of 2.0 mL/min. After the analytes (WR-1065 and WR-1729) elute, the mobile phase is changed via a linear gradient over 15 minutes to 90:10 acetonitrile/water to flush the column. Reequilibration is with the analytical mobile phase via a linear gradient prior to continuation of analyses. The condition of the Hg/Au electrode was evaluated by daily standard injections. If in-

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* IBM Instruments, Inc., Wallingford, CT.
† Bioanalytical Systems, Inc., West Lafayette, IN.
‡ Rheodyne, Incorporated, Cotati, CA.
§ Whatman, Inc., Clifton, NJ.

adequate detector sensitivity or erratic detector response was noticed, a freshly coated Hg/Au cell was introduced and allowed to equilibrate overnight before further analyses were performed. In practice a cell may remain in good working condition for up to 2 weeks at which time it is routinely recoated and replaced.

Reagents

Acetonitrile and methanol were HPLC reagent grade.** Chloroacetic acid (99%) and dl-10-camphorsulfonic acid (98%)†† were purchased and used without further purification. All other chemicals were reagent grade quality. WR-1065, BN-BK05030 and WR-2823 [S-2-(5-aminopentylamino)ethyl phosphorothioate] AV 25574 were furnished by Walter Reed Army Institute of Research. WR-1729 [N-(2-mercaptoethyl)1,5-diaminopentane] was prepared from WR 2823 by acid hydrolysis following the method of Tabachnik *et al.*[5] for the conversion of WR-2721 [S-2-(3-aminopropylamino)ethyl phosphorothioate] to WR-1065.

Under a stream of purified nitrogen a solution of 25 mg of WR-2823 in 25 mL of 1 N HCl was heated to boiling and gently refluxed for 5 minutes. The resultant HCl solution of the hydrolysis product (WR-1729) was cooled and stored frozen at −20°C.

Standards containing WR-1065 were prepared by dissolution of the solid in 0.1 M chloroacetic acid. Standards containing WR-1729 were prepared by volumetric dilution of the stock WR-1729 solution using 0.1 M monochloroacetic acid. Calibration curves were constructed by plotting the ratio of peak height WR-1065/peak height WR-1729 versus the WR-1065 plasma concentration. Three curves were obtained representing the three internal standard fortification levels.

Sample preparation

WR-1729 internal standard solution (63.5 μL) is added to 100 μL of plasma contained in a 6-mL polyethylene centrifuge tube. If required, WR-1065 is added at this time. To the plasma solution is added 200 μL of 0.2 N perchloric acid and sufficient 0.1 N chloroacetic acid such that the final sample volume is 500 μL. The contents of the tube are mixed for 30 seconds using a Vortex mixer‡‡ and the tube then placed in an ice bath for 5 minutes. The precipitated protein is separated by centrifugation in a refrigerated centrifuge‡‡ at 2000 RPM for 5 minutes. Separated supernatant (20 μL) was injected via a stainless steel sampling loop onto the HPLC analytical column.

Alternatively, 100-μL portions of plasma, which had been stored frozen in separate 6-mL polyethylene tubes at −78°C, were individually thawed and treated as described above. Internal standard had not been added prior to freezing.

Table 1. Statistics of WR-1065 standard curves

	Plasma levels of WR-1065, μg/ml		
Concentration of internal standard, μg/ml*	1–10 7.5	10–100 75	100–500 750
Slope of standard curve†	0.26	0.024	0.0016
Intercept of standard curve	−0.084	0.19	0.42
Coefficient of determination	0.9945	0.9950	0.9737
Degrees of freedom	3	3	2

* WR-1729, N-(2-mercaptoethyl)-1,5-diaminopentane.

† Linear regression of (peak height of WR-1065)/(peak height of WR-1729) against concentration of WR-1065, μg/ml.

Animal dosing experiments

A healthy, 1 year-old AKC registerable male Beagle dog weighing 15.4 kg was used in a pilot dosing experiment to test the analytical method. The animal was dosed intravenously in the cephalic vein with a solution of 2.32 g of WR-2721 in 0.9% saline solution (150 mg/kg body weight). The infusion required 2 minutes. Blood samples (3 mL) were withdrawn into an EDTA containing Vacutainer from a cannula placed in the jugular vein. Each sample was immediately chilled in ice water and centrifuged to separate the plasma. Portions of the chilled plasma (90 μL) were placed in polyethylene centrifuge tubes, frozen by immersion in a Dry Ice/isopropanol bath, and stored at −78°C until the time of analysis when they were individually thawed at room temperature as described in the Sample Preparation section.

RESULTS

Several candidate internal standard compounds were screened for their applicability to this analysis; however, only two, WR-1729 and the mercaptan hydrolysis product of WR-80855 [N-(3-mercaptopropyl)-1,4-diaminobutane], had appropriate retention characteristics. WR-1729 was chosen because its absolute retention time of 12 minutes was nearest to that for WR-1065, which was 9 min-

Table 2. Precision estimates for WR-1065 assay using WR-1729 as an internal standard

	Plasma levels of WR-1065, μg/ml		
	5.0	50	500
Number of replicates	5	5	5
Average peak height ratio	1.35	1.402	1.25
Standard deviation	0.037	0.039	0.048
Coefficient of variation, %	2.74	2.78	3.86
Internal standard concentration, μg/ml	7.5	75	750

** J. T. Baker Chemical Company, Phillipsburg, NJ.

†† Aldrich Chemical Company, Inc., Milwaukee, WI.

‡‡ American Scientific Products, McGraw Park, IL.

Table 3. Accuracy of WR-1065 analytical method

Spike level μg/ml	Measured level μg/ml	Percent deviation (D)
2.5	2.2	−12.0
7.5	7.1	−5.3
35	38.3	9.4
80	81.9	2.4
125	120	−4.0
375	389	3.7

Average deviation = |D|/n = 6.1.

utes. The mercaptan of WR-80855 did not elute until 19.4 minutes. WR-1065 and WR-1729 are 100% resolved under the assay conditions.

The linearity of the detector response over the assay range was investigated and three separate calibration curves were constructed to keep the level of the added internal standard near the plasma WR-1065 levels. Table 1 presents the statistics for the three standard curves.

The method precision was calculated by the analysis of replicate fortified plasma samples and has a coefficient of variation (CV) of less than 4% (Table 2) from 5 to 500 μg/mL WR-1065. The accuracy of the method (Table 3) was evaluated by the analysis of "unknown" fortified samples and has an average deviation of approximately 6% over the range of 2.5 to 375 μg/mL WR-1065.

The applicability of the method to the analysis of actual samples is shown in Figure 1. A male Beagle dog received an intravenous dose of 2.32 g (150 mg/kg) of WR-2721. Blood samples were obtained over time and the separated plasma analyzed for the WR-2721 hydrolysis product WR-1065. Figure 2 shows the chromatogram obtained from the analysis of the 1 minute post-infusion plasma

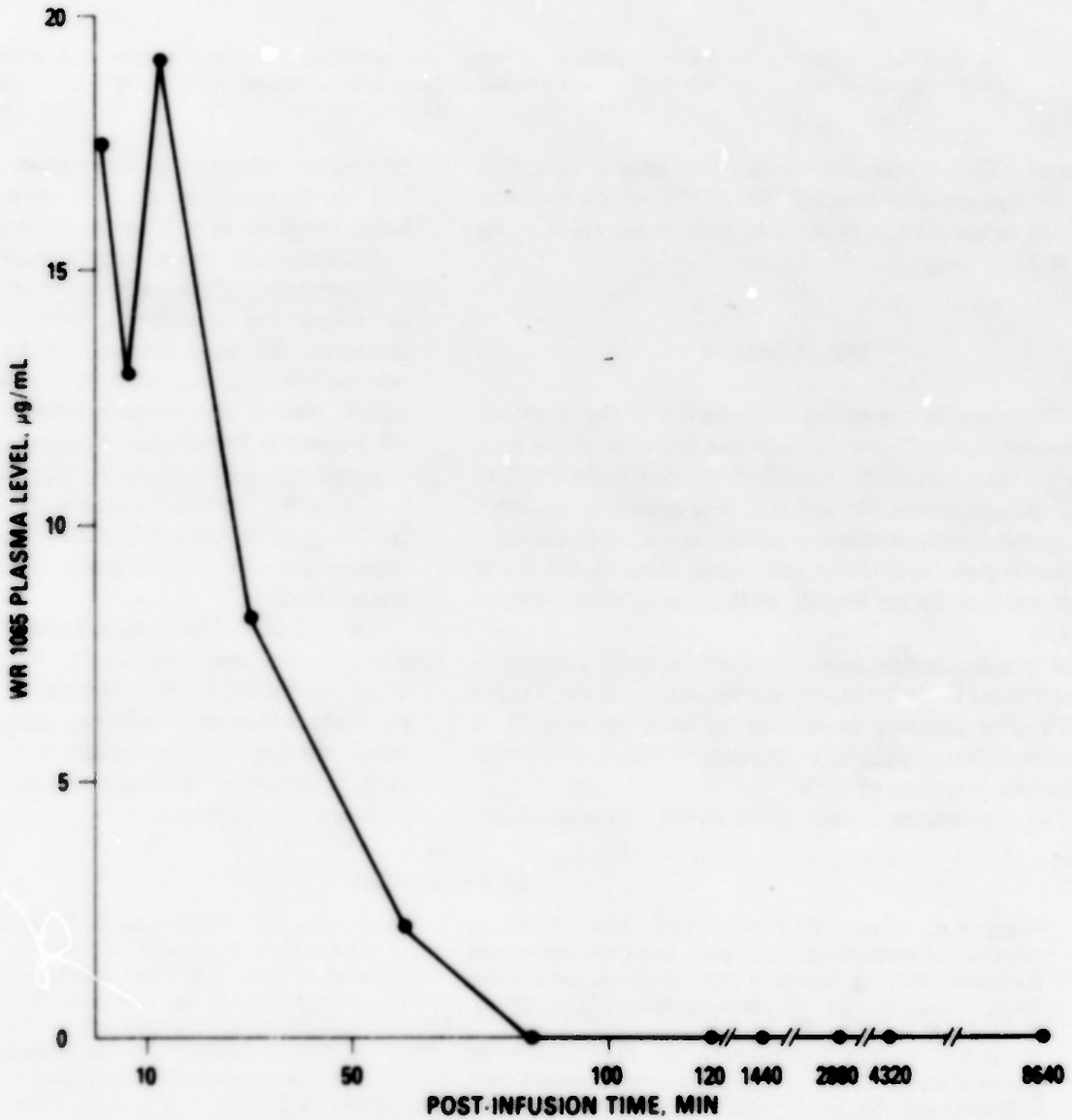

Fig. 1. Beagle plasma WR-1065 concentration versus time post-infusion for an IV WR-2721 dose of 2.32 g (150 mg/kg).

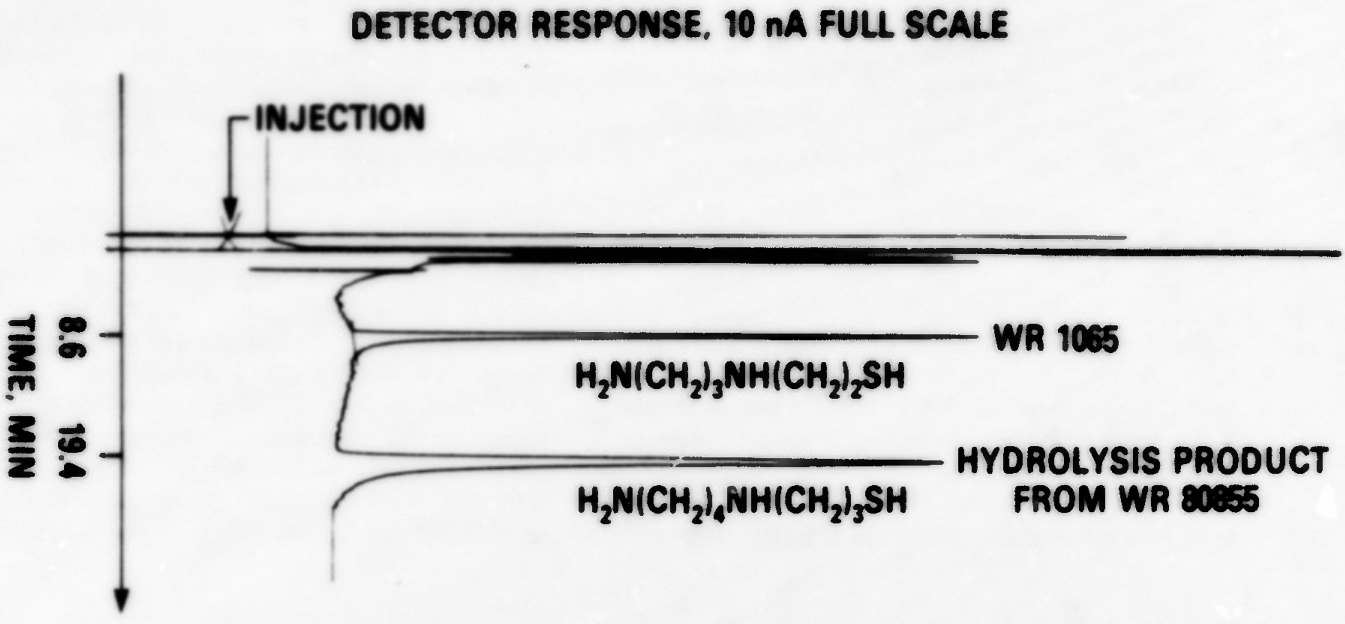

Fig. 2. HPLC chromatogram from the analysis of beagle plasma sample obtained 1 minute post-infusion of 2.32 g (150 mg/kg) of WR-2721. WR-80855 has been added as an internal standard for the WR-2721 assays.

sample. The second chromatographic peak is attributed to the hydrolysis product of WR-80855, which had been added to the plasma to serve as an internal standard for WR-2721 assays.

DISCUSSION

The statistics describing the behavior of the three calibration curves (Table 1) show the linearity of the peak height ratio versus concentration function within each of the defined ranges ($R^2 < 0.97$). It is apparent, however, from the slope and intercept differences between the three linear regions that the overall curve shape is not linear but tends to flatten slightly at the higher concentration levels.

The assay precision is good over the entire concentration range (Table 2) with the coefficient of variation being <4%. The accuracy as determined from the analysis of fortified plasma samples is satisfactory having an average absolute deviation of 6.1%.

No derivatization steps are involved and preparation for analysis requires only a minimum of sample handling. The Hg/Au electrode offers the advantage of being both highly selective as well as being very sensitive giving a quantifiable response to approximately 1 ng of thiol.

The results of the analysis of the plasma obtained from the Beagle dog after dosing with WR-2721 (Figure 1) dramatize the rapid decrease in the plasma WR-1065 concentration from a maximum concentration of 19.1 μg/mL plasma at 6 minutes post-infusion to <0.3 μg/mL plasma at 84 minutes. Analysis of samples obtained at greater post-infusion times failed to indicate the presence of WR-1065. These observations suggest that WR-1065 may be forming mixed disulfides with endogenous plasma components and rapidly being removed from the plasma pool.

An extension of the present analytical protocol to allow detection and quantification of disulfides using a dual series Hg/Au electrode configuration with the upstream electrode acting as a reducing agent generating thiols, which are then instantaneously detected and quantified at the downstream electrode, will allow this possibility to be more fully explored.

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APPENDIX F

Protocols and Logs
Dosing Studies No. 4 thru 8

January 17, 1983 Study No. 4

Preliminary Study of Intravenous WR 2721 Pharmacokinetics Using a New HPLC Assay

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intravenous dose of the drug. Plasma samples will be split and assayed on two separate occasions to develop specifications for day-to-day variations in assayed samples.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment. (Note 5)

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited.

An indwelling catheter for drug administration will be placed in one of the cephalic veins. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved in a final concentration of 100 mg/mL WR 2721 or 125 mg/mL WR 2721·3H_2O. The drug will be administered over two (2) minutes and the catheter will be flushed with 3-5 mL of normal saline.

A blood sample (10 mL) will be collected in EDTA vacuotainers before dose injection and blood samples (3 mL) at 1, 2, 3, 4, 6, 9, 15, 22, 30, 40, 50, 60, 72, 75, 84, 96, 108, 120, 180, 240, 300, 360, 480, 1440 minutes post-injection. The end of the infusion period (2 min) is set as time zero. The blood samples will be collected immediately cooled in ice water, centrifuged, divided into two aliquots, spiked with internal standard and

stored frozen prior to analysis (Note 6). The blood in the catheters will be replaced with heparinized saline (Note 7). The catheters will be removed at the end of the experiment.

One complete set of each plasma samples will be assayed on two separate occasions to give information on the day-to-day assay variation.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. [Dose for 14.09 kg (31 lb) dog will be 2.11 g of WR 2721 on a nonhydrated basis (2.64 g as the trihydrate). Total dosage formulation prepared should be 3.750 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Dose would be 21.1 mL.]

2. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of approx. 1500 µg/mL assuming total blood of dog is 1.5 L.

3. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

4. WR 2721 dose will be prepared on date of dosing study. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe.

5. Dog should be fed once/day preferrable in afternoon (3-4 p.m.) to better accomodate dosing study. Dog should be on regular diet.

6. Blood sample immediately after being drawn will be spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice-isopropyl alcohol (-72°C) and forwarded to Building 70 for storage at -20°C until assayed. Detailed written procedures will be prepared for the assay.

7. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

Beagle Dog (6223-B-1) Dosing Study - 1-19-83

Protocol followed dated January 17, 1983
(copy attached)

Dog Weight: 30.0 lbs (13.64 kg)

Dose: 20.5 mL of WR 2721 solution at 125 mg/mL of WR 2721 trihydrate equivalent to 150 mg/kg of WR 2721 (anhydrous)

TOTAL DOSE WEIGHT: 2.046 g (anhydrous WR 2721)
2.56 g WR 2721 $3H_2O$

Dose Infusion Time: 2 minutes, time started after total dose infusion

Blood Samples: 0 time (15, 31, 35, 40, 45, 50, 55, 60, 65, 72, 75, 84, 96, 109, 120, 180, 240, 300, 360, 420, and 1440 (post injection) also 7200 min (5 days).

NOTES:

1. Difficulty with catheter prevented all samples be taken at times suggested in protocol. Some extra samples were therefore taken.

2. New and different vacuotainers were used. Liquid EDTA solution was noted as being yellow colored.

3. Sample taken at 1440 min was taken in different vacuotainer having solid EDTA.

Beagle Dog (6223-B-1) Dosing Study - 1-19-83 Study No. 4

Time, min.	Start	Finish	Remarks
0 time	9:14 AM	9:15 AM	
Dosing	9:27 AM	9:29 AM	
1			no samples taken.
2			catheter out
3			"
4			"
5			"
6			"
9			"
15	15:00 min	15:23	vomiting
18			no samples taken
22			catheter out
26			
30(31)	30:87	31.00	
35	35:00	35:33	dog tranquil
40	40:00	40:40	
45	45:00	45:23	
50	50:00	50:24	
55	55:00	55:20	
60	60:00	60:20	
65	65:00	65:20	
72	72:00	72:20	
75	75:00	75:20	
84	84:00	84:25	dog took water 86:00 min
96	96:00	96:26	dog vomited 100cc 87:00 min
109	109:00	109:25	
120	120:00	120:23	
180	180:00	180:24	
240	240:00	240:120	2:00 PM dog took 150-200 mL H_2O
300	300:00	300:17	
360	360:00	360:74	
420	420:00(?)	425:40	catheter clotted
1440	1440·00		sample collected using syringe: different vacuotainer

February 16, 1983 (Revised) Study No. 5

Preliminary Study of Intravenous WR 2721 Pharmacokinetics Using a New HPLC Assay

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intravenous dose of the drug. Plasma samples will be split and assayed by two different analytical procedures to compare results.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment. (Note 5)

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited. (Note 4)

An indwelling catheter for drug administration will be placed in one of the cephalic veins. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved to give a final concentration of 100 mg/mL WR 2721 or 125 mg/mL WR 2721·$3H_2O$. The drug will be administerd over two (2) minutes and the catheter will be flushed with 3-5 mL of normal saline.

A blood sample (10 mL) will be collected in EDTA Vacutainer® before dose injection and blood samples (3 mL) at 1, 2, 3, 6, 9, 15, 22, 30, 40, 50, 60, 72, 75, 84, 96, 108, 120, 180, 240, 300, 360 minutes and 24, 30, 48 and 120 hours post-injection. The end of the infusion period (2 min) is set as time zero. The blood samples will be collected immediately cooled in ice water, centrifuged, divided into aliquots, spiked with internal

standards and stored frozen prior to analysis (Note 6). The blood in the catheters will be replaced with heparinized saline (Note 7). The catheters will be removed at the end of the experiment.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 15 kg dog will be 2.25 g of WR 2721 on a nonhydrated basis (2.81 g as the trihydrate). Total formulation prepared will be 3.750 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Dose would be 22.5 mL.]

2. Formulation of dose will be within one hour prior to infusion time.

3. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of ~ 1500 µg/mL assuming total blood of dog is 1.5 L.

4. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

5. WR 2721 dose will be prepared within one hour of dosing. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe.

6. Dog will be fed once/day in afternoon (3-4 p.m.) to better accomodate dosing study. Dog should be on regular diet.

7. Blood sample immediately after being drawn will be cooled in ice water and spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice-isopropyl alcohol (-72°C) and forwarded to Building 70 for storage at -20°C until assayed. Detailed written procedures will be prepared for the assay.

8. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

DOG DOSING STUDY Study No. 5 DATE: 2/16/83

Dog Breed: Beagle #409 Dog Weight: 13.64 kg (30 lbs) Protocol Date: 2/16/83 Revised

Dose WR 2721: 20.5 mL of WR 2721·3 H_2O (125 mg/mL)

2·557 g (·3H_2O); 2·0455 (WR 2721) 150 mg (kg weight)

TIME min	START	FINISH	REMARKS
Time	9:40 AM	9:42 AM	10 mL blood
Dosing	10:00 AM	10:02 AM	
1			Missed; poor blood flow.
2	1:90 min	2:00 min	Good blood flow.
3	3:40	3:60	Wretching
6	6:00	6:16	OK blood flow.
9	9:00	9:15	10:10 Emesis
12	12:00	12:20	
15	15:00	15:18	Vomiting
			Vomiting 21:50
22	22:00	22:15	23:20 Vomiting
			28:20 Vomiting
30	30:00	30:20	Dog tranquil
40	40:00	40:25	
50	50:00	50:20	
60	60:00	60:20	
72	72:00	72:25	
75	75:00	75:16	
84	84:00	84:25	
96	96:00	96:18	
108	109:00	109:18	Missed time by 1 minute.
120	120:00	120:18	
180	180:00	180:50	
240	240:00	240:24	
300	300:00	301:20	
360	360:00	360:20	
23 hrs	9:00 AM	(2/17/83)	
29 hrs	3:00 PM	(2/17/83)	
47 hrs	9:00 AM	(2/17/83)	
119 hrs	9:00 AM	(2/21/83)	

NOTES:

October 4, 1983 Study No. 6

Preliminary Study of Intravenous WR 2721 Pharmacokinetics Using a New HPLC Assay

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intraveneous dose of the drug. Plasma samples will be split to allow for a future WR 1065 assay as well as the WR 2721 assay.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment (Note 5).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited (Note 4).

An indwelling catheter for drug administration will be placed in one of the cephalic veins. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved to give a final concentration of 100 mg/mL WR 2721 or 125 mg/mL WR 2721·3H_2O. The drug will be administered over two (2) minutes and the catheter will be flushed with 3-5 mL of normal saline.

A blood sample (~~10~~ 3 mL) will be collected in EDTA Vacutainer® before dose injection and blood samples (3 mL) at 1, 2, 3, 6, 9, 12, 15, 22, 30, 40, 50, 60, 72, 75, 84, 96, 108, 120, 180, 240, 300, and 360 minutes and 24, 30, 48, 72 and 144 hours post-injection. The end of the infusion period (2 minutes) is set as time zero. The blood samples will be collected, immediately cooled in ice water, centrifuged, divided into aliquots, spiked with internal standards and stored frozen at -78°C prior

to analysis (Note 7). The blood in the catheters will be replaced with heparinzed saline (Note 8). The catheters will be removed at the end of the experiment.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 15.455 kg (34 lb) dog will be 2.32 g of WR 2721 on a nonhydrated basis (2.90 g as the trihydrate). Total formulation prepared will be 3.750 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Dose would be 23.2 mL.

2. Formulation of dose will be within one hour prior to infusion time.

3. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of ~ 1500 µg/mL assuming total blood of dog is 1.5 L.

4. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

5. WR 2721 dose will be prepared within one hour of dosing. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe.

6. Dog will be fed once/day in the afternoon (3-4 p.m.) to better accommodate dosing study. Dog should be on regular diet.

7. Blood sample immediately after being drawn will be cooled in ice water and spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice-isopropyl alcohol (-78°C) and forwarded to Building 70 for storage at 78°C until assayed. Detailed written procedures will be prepared for the assay.

8. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

DM/bz/I5

DOG DOSING STUDY Study No. 6 **DATE:** 10-4-83

Dog Breed: Beagle Dog Weight 14.455kg (34 lbs) Protocol Date: 10-4-83

Dose WR 2721: Dog Beagle No. 1 - 23.2 mL of WR 2721•$3H_2O$ (125 mg/mL); 2.90 g (•$3H_2O$); 2.32 g (WR 2721; 150 mg/kg by dog weight

TIME (min) 9:24 AM Time 9:26 AM	START	FINISH	REMARKS
Dosing			2 Min Infusion
1	1.0	1.55	
2	2.0	2.20	Vomiting
3	3.0	3.93	Nausea - no flow
6	6.0	6.80	Wretching 1/2 sample
9	9.0	9.3	Vomiting; wretching
12	12.35	12.45	Vomiting; wretching
15	15:50	15:60	
22	22.10	22.45	1/2 sample; slow fill
30	30.00	30.15	Vomiting; wretching: Relaxed
40	40.00	40.10	Tranquil
50	50.00	50.40	
60	60.00	60.15	Tranquil
72	72.00	72.25	approx. 3/4 sample; poor flow
75	75.00	75.10	
84	84.00	84.10	
96	96.00	96.10	Drank Water
108*	111.50	111.70	*Delayed sample
11:26 AM 120	120.00	120.40	Wretching (some)
12:26 PM 180	180.00	180.15	uneventful
1:26 PM 240	240.00	240.08	
2:26 PM 300	300.00	300.64	
3.26 PM 360	360.00	300.10	
10/5 9:26 AM 1440 (24 hr)	1442	1442.2	Draw 3 cc syringe from right arm
10/5 3:26 AM 1800 (30 hr)	1796	1796.2	uneventful (5 mL)
10/6 9:26 PM 2880 (48 hr)	2880	2880.4	
10/7 9:26 AM 4320 (72 hr)	4320	4320.2	
10/10 9:26 AM 8640 (144 hr)	8641	8641.3	

NOTES: Dose prepared 8-8:30 AM 10/4/83

Blood sample taken at 9:20 AM (3 mL) difficulty in drawing sample

October 26, 1983 (Revised) Study No. 7

PRELIMINARY STUDY OF INTRAVENOUS WR 2721 PHARMACOKINETICS USING A NEW HPLC ASSAY

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intraveneous dose of the drug. Plasma samples will be split to allow for a future WR 1065 assay as well as the WR 2721 assay.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment (Note 6).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited (Note 4).

Infusion will be by needle and syringe or infusion pump. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved to give a final concentration of 100 mg/mL WR 2721 or 125 mg/mL WR 2721·$3H_2O$. The drug will be administered over 10 minutes.

A blood sample (10 mL) will be collected in EDTA Vacutainer® before dose injection and blood samples (3 mL) at 2, 3, 4, 6, 9, 12, 15, 22, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 180, 240, 300, 360 and 480 minutes and 12, 16, 24, 30, 48, 54, 120 and 144 hours post-injection. The end of the infusion period (10 minutes) is set as time zero. The blood samples will be collected, immediately cooled in ice water, centrifuged, divided into aliquots, spiked with internal standards and stored frozen at -78°C

prior to analysis (Note 7). The blood in the catheters will be replaced with heparinzed saline (Note 8). The catheters will be removed at the end of the experiment.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 13.64 kg (30 lb) dog will be 2.05 g of WR 2721 on a nonhydrated basis (2.56 g as the trihydrate). Total formulation prepared will be 3.750 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Dose would be 20.5 mL.

2. Formulation of dose will be within one hour prior to infusion time.

3. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of approximately 1500 µg/mL assuming total blood of dog is 1.5 L.

4. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

5. WR 2721 dose will be prepared within one hour of dosing. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe.

6. Dog will be fed once/day in the afternoon (3-4 p.m.) to better accommodate dosing study. Dog should be on regular diet.

7. Blood sample immediately after being drawn will be cooled in ice water and spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice-isopropyl alcohol (-78°C) and forwarded to Building 70 for storage at 78°C until assayed. Detailed written procedures will be prepared for the assay.

8. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

DM/bz/K4

Study No. 7

DOG DOSING STUDY Beagle 01-6223-B-2 Tatoo BD13 (Left Ear) DATE: 10-26-83

Dog Breed: Beagle-2 Dog Weight 13.64 kg (30 lbs) Protocol Date: 10-26-83 Revised

Dose WR 2721: 20.5 mL 2.05 g WR 2721; 2.56 as WR 2721• $3H_2O$

9:25 AM; 6 mL blood drawn

TIME (min)	START	FINISH	REMARKS
Time	Min	Min	
Dosing (9:26 AM)	(9:26 AM)	(9:36 AM)	Infusion 10 min. using needle and syringe
2	2:00	2:10	
4	4:00	4:98	Syringe used; Blood flow prob*
6	6:30	6:64	Slower withdrawal
9	9:00	9:22	Salvating
12	12:10	12:24	
15	15:10	15:34	Dog excited; 19 min tranquil
22	22:00	22:40	Wretching and vomited
30	30:00	30:20	
40	40:00	40:16	
50	50:00	50:15	Vomiting
60	60:00	60:10	
70	70:00	70:05	Shivering
80	80:00	80:06	
90	90:00	90:05	
100	100:0	100:05	
110	110:0	110:04	
120	120:0	120:05	
180	180:0	180:04	
240	240:0	240.04	
300	300:0	300:08	
360	360:0	360:05	
480 (8 hr)	480:0	480:10	
720 (12 hr)	720:0	720:06	Remove jugular cath.
960 (16 hr)	960:0	960:08	Remove leg cath.
1440 (24 hr)	440:0	440:06	Draw directly from vein.
1800 (30 hr)	1800	1800:08	" "
2880 (48 hr)	2880	2880:10	" "
3240 (54 hr)	3240	3240:20	" "
7260 (121 hr)**	7266	7266:20	
8580 (143 hr)***	8615	8615:20	

NOTES: Wretching 8:20 sec into injection/9:25 Vomiting

*Early problem with vacutainer was probably due to vomiting and neck muscles drawn up which kinked the indwell cath in jugular. Vacutainer worked excellent after ca 90 min.

**Time change + 1 hour.

***1 hour early.

December 14, 1983 Study No. 8

PRELIMINARY STUDY OF INTRAVENOUS WR 2721 PHARMACOKINETICS USING A NEW HPLC ASSAY

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intraveneous dose of the drug. Plasma samples will be split to allow for a future WR 1065 assay as well as the WR 2721 assay.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment (Note 6).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited (Note 4).

Infusion will be by needle and syringe or infusion pump. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved to give a final concentration of 100 mg/mL WR 2721 or 125 mg/mL WR 2721·3H_2O. The drug will be administered over 10 minutes.

A blood sample (6 mL) will be collected in EDTA Vacutainer® before dose injection and blood samples (3 mL) at 2, 4, 6, 8, and 10 minutes after the start of infusion period and at 2, 3, 4, 6, 9, 12, 15, 22, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 180, 240, 300, 360 and 480 minutes and 12, 16, 24, 30, 48, 54, 120 and 144 hours post-injection. The end of the infusion period (10 minutes) is set as time zero. The blood samples will be collected, immediately cooled in ice water, centrifuged, divided into

aliquots, spiked with internal standards and stored frozen at -78°C prior to analysis (Note 7). The blood in the catheters will be replaced with heparinzed saline (Note 8). The catheters will be removed at the end of the experiment.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 15.69 kg (34.5 lb) dog will be 2.36 g of WR 2721 on a nonhydrated basis (2.94 g as the trihydrate). Total formulation prepared will be 3.750 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Dose would be 23.6 mL.

2. Formulation of dose will be within one hour prior to infusion time.

3. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of approximately 1500 µg/mL assuming total blood of dog is 1.5 L.

4. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

5. WR 2721 dose will be prepared within one hour of dosing. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe.

6. Dog will be fed once/day in the afternoon (3-4 p.m.) to better accommodate dosing study. Dog should be on regular diet.

7. Blood sample immediately after being drawn will be cooled in ice water and spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into portions, as appropriate for assay, the samples frozen in Dry Ice-isopropyl alcohol (-78°C) and forwarded to Building 70 for storage at 78°C until assayed. Detailed written procedures will be prepared for the assay.

8. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

9. Dog temperature during study should be measured rectally and recorded. Dog should be kept covered and on wooden base to maintain body temperature.

10. Note change in sampling during infusion period.

DM/bz/P4

DOG DOSING STUDY Study No. 8 DATE: 12-14-83

Dog Breed: Beagle 8-1 Dog Weight 15.69 kg (34.5 lbs) Protocol Date: 12-14-83

Dose WR 2721: 2.36 g WR 2721 (2.94 g WR 2721·$3H_2O$)
23.6 mL of 125 mg/mL
6 mL blood for baseline 9:45 am 38.6°C (100.5°F)

TIME (min)	START	FINISH	RECTAL TEMPERATURE	REMARKS
Time	0.00	8.80	38.6°C	
Dosing				
A 2	2.00	2.30	38.6°C	Small sample
A 4	4.00	4.40	38.6°C	Salivating
A 6	6.00	6.26		Vomiting - heaves
A 8	8.00	8.10		
A 10	Not taken			
P 2	11.10	11.22		Wretching
P 4	13.00	13.08	38.6°C	Vomiting
P 6	15.00	15.30	39.0°C	Vomiting; slight blood sample
P 10	19.00	19.30		Vomiting
P 12	21.00	21.13	39.0°C	(100°F on sec. run)
P 15	24.00	24.15	100°F	Wretching
P 22	31.00	31.10	100°F	Tranquil
P 30	39.00	39.10	99.5°F	Tranquil covered - on board
P 40	49.00	49.10	99.0°F	Tranquil
P 50	59.00	59.15	99.0°F	Tranquil
P 60	69.00	69.20	99.0°F	Tranquil
P 70	79.00	79.45	98.5°F	Syringe taken; tranquil
P 80	89.00	89.10	98.5°F	Tranquil
P 91	100.50	100.60	98.5°F	Tranquil
P 100	109.00	109.08	98.5°F	Tranquil
P 110	119.00	119.10	98.5°F	Tranquil - took water
P 121	130	130.40	98.5°F	Shivering - alert
P 180	189	189.7	99.20°F	Sleeping
P 240	249	249.4	99.50°F	Tranquil
P 300	309	309.2	100.00°F	Tranquil
P 360	369	369.8	99.00°F	Tranquil
P 480 (8 hr)	487	487.5	100.00°F	Tranquil
P 720 (12 hr)	728	728.2	99.00°F	Slight shivering
P 960 (16 hr)	967	967.2	99.00°F	Slight shivering
P 1440 (24 hr)	1443	1444	100.8°F	Slight shivering
P 1800 (30 hr)	1807	1807.2	101.4°F	Normal
P 2880 (48 hr)	2887	2887.5	-	(No temp taken)
P 3240 (54 hr)	3250	3251	100.05°F	Thermistor probe
P 7200 (120 hr)	7214	7214.6	-	(No temp taken)
P 8640 (144 hr)	8647	8649.5	102.2°F	Normal

NOTES: 1. For future: Draw samples by syringe initially because of wretching
2. Infusion completed at 9.0 minutes
3. Thermistor probe rectally for temperature thru P960 sample.
Rectal thermometer used thereafter except P3240.

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SUPPLEMENTARY

INFORMATION

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

REPLY TO
ATTENTION OF:

ERRATA

SGRD-RMI-S (70-1y) B094 846

31 JUL 1992

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Bldg. 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Development Command (USAMRDC), has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-80-C-0128. Request the limited distribution statement for AD Nos. ADB063672, ADB080528, ADB094912, [redacted], ADB109071, and ADB109038, be changed to [redacted] distribution unlimited," and that copies of these reports be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller, DSN 343-7325.

ERRATA

CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

CF:
Dr. Ruthann Smejkal/WRAIR

Errata: B094 846